# Amoebic Gill Disease of Atlantic Salmon: Resistance, Serum Antibody Response and Factors That May Influence Disease Severity

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## Declaration of originality

This thesis contains no material which has been accepted for a degree or diploma by this university or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis.

Benita Vincent

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## Abstract

Amoebic gill disease (AGD) is a condition of some marine-cultured fish worldwide and is the result of *Neoparamoeba* spp. infection. If AGD-affected fish are left untreated, major mortalities can occur. In Tasmania, Australia, fresh water bathing remains the only treatment for AGD, a practice that represents approximately 10-20% of production costs. Therefore, development of a preventative measure such as vaccination is a priority for Tasmanian salmon growers. In this project, resistance of Atlantic salmon to AGD and the development of a serum antibody response to *Neoparamoeba* spp. were assessed. Sera from AGD-affected Atlantic salmon were screened to identify potential candidate antigens for an AGD vaccine. Atlantic salmon exposed to *Neoparamoeba* spp. and subsequently challenged with AGD demonstrated resistance in terms of increased survival compared to AGD-naïve fish. In addition, antibodies that bound cell-surface carbohydrate epitope(s) of *Neoparamoeba* spp. were detected in the sera of some fish after secondary exposure to *Neoparamoeba* spp.. In light of this, further screening of sera from Atlantic salmon exposed to *Neoparamoeba* spp. in the laboratory or during sea-cage culture was conducted. Antibodies present in the sera of some AGD-affected Atlantic salmon predominately bound carbohydrate residues expressed on the cell-surface of *Neoparamoeba* spp..

Ideally an AGD vaccine would contain peptide antigen(s) that can easily be produced by recombinant DNA technology. Therefore, an alternative approach to identify candidate vaccine antigens for an AGD vaccine was needed. Some pathogenic amoebae colonise

host tissues via lectin-mediated attachment and lectins have shown promise as candidate vaccine antigens. The *in vivo* effect of mucus and saccharides on the ability of *Neoparamoeba* spp. to cause AGD was investigated. The number of AGD lesions was significantly reduced when amoebae were incubated in mucus or any of the range of saccharides assessed. These data suggest that colonisation of *Neoparamoeba* spp. on gill tissues of Atlantic salmon may be lectin-mediated and the ensuing infection can enhance resistance to *Neoparamoeba* spp.. However, very few AGD-affected Atlantic salmon develop a serum antibody response to *Neoparamoeba* spp. suggesting that the development of antibody-mediated protection of Atlantic salmon during *Neoparamoeba* spp. infection is unlikely.

**Note to the reader**

Each research chapter presented in this thesis was prepared as a stand-alone piece of work in view of submitting each chapter for publication. As a result, there is a level of unavoidable repetition in the Introduction and Materials and Methods sections of the research chapters. The referencing style of this thesis follows the style of the Journal of Fish Diseases.

AGD is caused by *Neoparamoeba perurans.* However it has been shown that a range of amoebae can be isolated from gills of AGD-affected Atlantic salmon. As my work utilises gill-isolated amoebae, it is likely that the preparations contain *N. perurans* and other *Neoparamoeba species*. Therefore, throughout this thesis I refer to gill–isolated amoebae as *Neoparamoeba* spp..

# Chapter 1

# General Introduction

**Amoebic gill disease of Atlantic salmon**

Amoebic gill disease (AGD) predominately affects sea-caged Atlantic salmon, *Salmo salar* L., and was first described by Munday (1986) shortly after Atlantic salmon culture began in Tasmania. In southern Tasmania, AGD is most prevalent during summer months in association with water temperatures in excess of 15°C and salinity of 35 ‰ (Kent, Sawyer & Hedrick, 1988; Clark & Nowak, 1999; Adams & Nowak, 2003). AGD of salmonids has also been reported in Ireland (Rodger & McArdle, 1996; Palmer, Carson, Ruttledge, Drinan & Wagner, 1997), the USA (Kent, et al., 1988), Chile, New Zealand (Munday, Zilberg & Findlay, 2001), Scotland (Young, Dyková, Snekvik, Nowak & Morrison, 2007b) and Norway (Steinum, Kvellestad, Rønneberg, Nilsen, Asheim, Fjell, Nygård, Olsen and Dale). AGD also affects marine-farmed turbot, *Psetta maxima* L., (Dyková, Figueras & Novoa, 1995; Dyková, Figueras, Novoa & Casal, 1998) and *Neoparamoeba* spp. have been isolated from the gill tissues of moribund European sea bass, *Dicentrarchus labrax* L., from farms experiencing AGD (Dyková, Figueras & Peric, 2000).

Based on morphological observation, the aetiological agent of AGD was initially described as *Paramoeba pemaquidensis,* Page 1970, (Kent, et al., 1988; Roubal, Lester & Foster, 1989). However these gill-derived amoebae did not possess microscales (Kent, et al., 1988; Dyková, et al., 2000) as described for members of the Paramoebidae family and this species was re-assigned to the Vexilliferidae family (Page, 1987) and was subsequently referred to as *Neoparamoeba pemaquidensis*. Species of marine

*Neoparamoeba* are not known to have different life stages. Reproduction is by binary fission and *Neoparamoeba* spp. are not known to produce resting stages.

Molecular analysis of 18S rDNA from cultured strains of gill-isolated *Neoparamoeba* spp. provided further support for *N. pemaquidensis* as the causative agent of AGD (Wong, Carson & Elliot, 2004). However, as amoebae isolated from AGD-affected gill tissues of Atlantic salmon in Tasmania include *N. branchiphila* (Dyková, Nowak, Crosbie, Fiala, Pecková, Adams, Machácková & Dvoráková, 2005a) AGD was possibly a disease of mixed etiology. More recently, analysis of 18S and 28S rDNA from wild-type *Neoparamoeba* spp. contributed to the identification of a new species, *Neoparamoeba perurans* (Young, Crosbie, Adams, Nowak & Morrison, 2007a).

*N. perurans* is the predominant agent of AGD of Atlantic salmon (Young, et al., 2007b). Furthermore, molecular assessment of gill histology, using oligonucleotide *in situ* hybridisation probes, of marine cultured Atlantic salmon from Australia, Ireland, Scotland and the USA, Chinook salmon, *Oncorhynchus tshawytscha* Walbaum, from New Zealand, rainbow trout, *Oncorhynchus mykiss* Walbaum, from Australia and turbot from Spain confirmed the presence of *N. perurans* in association with AGD lesions (Young, et al., 2007a; Young, et al., 2007b). These data provide compelling evidence that *N. perurans* is the predominant aetiological agent of AGD globally. While there is a possibility that a range of *Neoparamoeba* species may be present in gill-isolated preparations, it is most likely that the majority of the gill-isolated amoebae are *N.*

*perurans* as this is the species consistently associated with AGD-affected gill tissue (Young, Dyková, Snekvik, Nowak & Morrison, 2008b). Throughout this thesis where analysis of the binding of serum antibodies to wild-type *Neoparamoeba* spp. was conducted, binding of serum antibodies to *N. Branchiphila* and *N. pemaquidensis* was assessed using clonal cultured strains of these amoebae.

While a range of *Neoparamoeba* species may be present in gill-isolated preparations, it is most likely that the majority of the gill-isolated amoebae are *N. perurans* as this is the species consistently associated with AGD-affected gill tissue (Young, et al., 2008). Throughout this thesis where analysis of the binding of serum antibodies to wild-type *Neoparamoeba* spp. was conducted, binding of serum antibodies to *N. Branchiphila* and *N. pemaquidensis* was assessed using clonal cultured strains of these amoebae.

While *N. pemaquidensis* are detectable by PCR in gill-isolated amoebae from AGD-affected Atlantic salmon (Young, et al., 2007), *in situ* hybridisation with species-specific 18S rRNA oligonucleotide probes identified only *N. perurans* in gill-isolated amoebae preparations (Young, et al., 2007; Young, et al., 2008).

Wild-type *Neoparamoeba* consistently elicit AGD in the laboratory (Attard, Crosbie, Adams and Nowak, 2006), and there is no indication that virulence is altered over time. *N. perurans* is the causative agent of AGD in the field globally and in the laboratory (Young, et al., 2007; Young, et al., 2008). Cultured gill-derived amoebae tested to date

do not cause AGD (Kent, Sawyer and Hedrick, 1988; Findlay, 2001; Morrison, Crosbie, Cook, Adams and Nowak, 2005; Vincent, Adams, Crosbie, Nowak and Morrison, 2007). There is a possibility that there may be differences between gill-isolated amoebae obtained from the laboratory and the field. There may also be differences between the relative abundence of different amoebae that can be isolated from gill tissues depending on season. However, these factors have not been studied. Assessment of these factors would be difficult due to the fact that the level of AGD is closely controlled in the field by fresh water bathing and a relatively low number of amoebae can be isolated from a single fish.

The characteristic gross sign of AGD is the presence of white, raised lesions. In the absence of other conditions that present similar gross gill anomalies, gross gill pathology is a reasonable indicator of AGD (Adams, Ellard & Nowak, 2004). However the assessment of gill histopathology is required for a diagnosis. Hyperplasia of gill epithelium juxtaposed to *Neoparamoeba* spp. trophozoites produces gill lesions by the fusion of secondary gill lamellae (Roubal, et al., 1989; Zilberg & Munday, 2000; Adams & Nowak, 2001). Following colonisation of wild-type *Neoparamoeba* spp. on the gill tissues of Atlantic salmon, infiltration of leukocytes can be observed in the surrounding gill tissues (Adams & Nowak, 2001; Adams & Nowak, 2003; Bridle, Morrison, Cupit Cunningham & Nowak, 2006). Lesions may progress along the filament and at times, lamellar fusion of up to 50% of the gill filament can be observed (Adams & Nowak, 2004b).

Fresh water bathing remains the only commercially viable treatment for AGD of Atlantic salmon. While fresh water bathing significantly reduces the abundence of amoebae present on the gills, *Neoparamoeba* spp. may not be completely eliminated. After bathing, *Neoparamoeba* spp. continue to proliferate and the gross sign of AGD may be observed within one week (Clark, Powell & Nowak, 2003). Currently, fresh water bathing contributes to up to 20% of production costs equating to $15-20 Million annually (Pheroze Jungalwalla, personal communication). Therefore development of an alternative to fresh water bathing, such as vaccination, is a priority for Tasmanian salmon growers. Fish can become resistant to parasitism and the induction of resistance of Atlantic salmon to AGD may provide relief from fresh water bathing.

**Resistance to ectoparasites in fish**

The development of resistance in fish against ectoparasites in terms of reduced parasitism may be observed over time. For example, a substantial decline in parasite abundance was seen over time as rainbow trout that developed resistance to the monogenean *Gyrodactylus derjavini* Mikailov, (Lindenstrom & Buchmann, 2000). Similarly, measured by a reduction in parasite abundance winter flounder, *Pseudopleuronectes americanus* Walbaum, demonstrated resistance to *Gyrodactylus pleuronecti* Cone, and/or *Trichodina murmanica* Poljansky, (Barker, Cone & Burt, 2002). Measured by survival, some Atlantic salmon demonstrated resistance to AGD after prolonged exposure to wild-type *Neoparamoeba* spp. (Bridle, Carter, Morrison & Nowak, 2005). Immunisation of

Atlantic salmon with wild-type *Neoparamoeba* spp. antigens administered by bath (Morrison & Nowak, 2005), or by intraperitoneal (i.p) injection (Zilberg & Munday, 2001) failed to elicit resistance to AGD.

In some cases, secondary exposure to the parasite is required before resistance is observed. Pompano, *Trachinotus marginatus* Cuvier, re-exposed to the monogenean *Bicotylophora trachinoti* MacCallum, displayed a reduced mean abundance of parasites after 30 days while the abundence of parasites on fish exposed a single time increased (Chaves, Luvizzotto-Santos, Sampaio, Bianchini & Martinez, 2006). Atlantic salmon developed resistance to AGD in terms of reduced gill pathology after secondary exposure to wild-type *Neoparamoeba* spp. (Findlay, Helders, Munday & Gurney, 1995; Findlay & Munday, 1998). However, in the study described by Gross, Morrison, Butler and Nowak, (2004b), previous exposure to wild-type *Neoparamoeba* spp. did not influence the survival or gill pathology of Atlantic salmon subsequently challenged with AGD. There were notable differences in the experimental designs applied in these studies. In the studies described by Findlay et al., (1995; 1998) water temperatures were maintained at 14°C compared to the 17°C applied by Gross et al., (2004a). In addition to salinity, water temperature is a key factor in the development of AGD (Clark & Nowak, 1999). The higher water temperature may have influenced results presented by Gross et al., (2004a) and while some resistance to *Neoparamoeba* spp. was observed in a group of Atlantic salmon that were maintained in sea water, the fresh water bath treatment failed to completely remove *Neoparamoeba* spp. from the gills. Therefore during the period

before challenge, AGD progressed resulting in ongoing mortalities prior to challenge, perhaps removing the most susceptible fish. Demonstration of resistance in Atlantic salmon to AGD must precede further study into the development of prophylactic measures. The development of resistance of Atlantic salmon to wild-type *Neoparamoeba* spp. requires further investigation and represents the starting point for this thesis. Resistance of fish to ectoparasites has, in some cases, been associated with the development of an antibody response, suggesting that development of adaptive immunity may be beneficial for guarding against ectoparasitic infestations.

**Development of an antibody response in fish to ectoparasites**

Parasite-specific antibodies have been detected in the serum and mucus of some fish after exposure to ectoparasites (Table 1.1). For example, bluegill sunfish, *Lepomis macrochirus* Rafinesque, developed serum and mucosal antibodies after repeated exposure to the larvae of the fresh water mussel *Utterbackia imbeciliis* (Rogers-Lowery, Dimock & Kuhn, 2007). Serum antibodies are detected in largemouth bass, *Micropterus salmoides* Lacepide, after repeat exposure to the broken rays mussel, *Lampsilis reeveiana* (Dodd, Barnhart, Rogers-Lowery, Fobian & Dimock, 2006). Modest antibody activity has been reported in serum from AGD-affected Atlantic salmon cultured in sea-cages (Gross, Carson and Nowak, 2004) and in the serum of fish exposed to wild-type *Neoparamoeba* spp. in the laboratory (Akhlaghi, Munday, Rough and Whittington, 1996). However in both cases, antibodies were detected after binding to antigens of cultured *N. pemaquidensis*. Cultured *Neoparamoeba* spp. assessed to date failed to elicit

AGD in Atlantic salmon (Kent, et al., 1988; Howard, Carson & Lewis, 1993; Findlay, 2001; Morrison, et al., 2005; Vincent, et al., 2007a). Therefore, antibody binding to cultured amoebae antigen(s) may not represent antigens expressed by wild-type *Neoparamoeba* spp. *in vivo.* The development of a serum antibody response in AGD-affected Atlantic salmon to wild-type *Neoparamoeba* spp. has not yet been determined.

The development of a serum antibody response to wild-type *Neoparamoeba* spp. is plausible. Amoebae entrapped within interlamellar cysts are often seen in association with leucocytes in gill sections of AGD-affected fish (Kent, et al., 1988; Munday, Foster, Roubal & Lester, 1990; Rodger & McArdle, 1996; Adams & Nowak, 2001; Bridle, Butler & Nowak, 2003; Dykova, Nowak, Crosbie, Fiala, Peckova, Adams, Machackova & Dvorakova, 2005). Interlamellar cysts provide the environment for interaction of *Neoparamoeba* spp. with immune cells (Fig. 1.1). Furthermore, antigen processing may occur at the site of infection as cells expressing MHC II have been observed throughout AGD lesions present in Atlantic salmon gill tissues (Morrison, Koppang, Hordvik & Nowak, 2006). Assessment of the serum antibody response of AGD-affected Atlantic salmon to wild-type *Neoparamoeba* spp. contributes the majority of work presented in this thesis.

The detection of antibodies has been reported for fish affected by ectoparasites. For example, an antibody response towards *Amyloodinium ocellatum* is detectable in the serum of tomato clown fish, *Amphiprion frenatus* Bloch, that demonstrate resistance to

*A. ocellatum* after repeated exposure (Cobb, Levy & Noga, 1998). Goldfish, *Carassius auratus* L., immunised with live *Ichthyophthirius multifiliis* or *Tetrahymena pyriformis* Lwoff, developed a parasite-specific serum and mucosal antibody response and were cross-protected against *Oodinium* spp., *Ichthyobodo* spp. or *Chilodonella* spp. (Ling, Sin & Lam, 1993). Rainbow trout immunised with *Discoctyle sagittata* developed a serum antibody response and demonstrated resistance in terms of reduced parasitism (Rubio-Godoy, Sigh, Buchmann & Tinsley, 2003b). Secondary exposure of largemouth bass to the molluscan parasite *Lampsilis reeveiana* conferred resistance in association with the development of a serum antibody response (Dodd, et al., 2006). Antibodies were detected in the serum of Atlantic salmon demonstrating resistance to wild-type *Neoparamoeba* spp. following secondary exposure, however antibodies were detected after binding to cultured *N. pemaquidensis* (Findlay & Munday, 1998). While in the majority of these cases, an antibody response has been induced by immunisation; parasite-specific antibodies can be detected after fish are infected (Table 1.1).

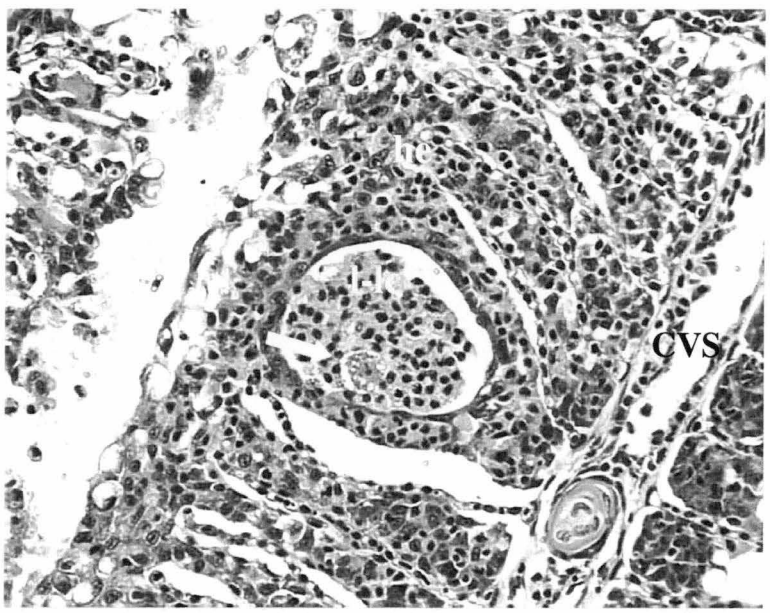


**Figure 1.1.** *Neoparamoeba* spp. trophozoites can become entrapped in interlamellar cysts where interaction with leucocytes may be observed. Histological section of gill tissues from an AGD-affected Atlantic salmon featuring an interlamellar cyst containing a *Neoparamoeba* spp. trophozoite (white arrow) surrounded by leucocyte-like cells (l-lc). Central venous sinus (CVS). Hyperplastic epithelium (he). Photo taken by the author.

**Table 1.1** Marine and fresh water fish can develop resistance to a range of parasites and in some cases this may be associated with an antibody response. Host resistance demonstrated after surface exposure or vaccination with ectoparasites, duration of exposure, the detection of an antibody response and the method used to assess presence of antibodies are summarised.

| Ectoparasite | Host | Host resistance[+] Exposure | Vaccination | Temperature (°C) | Duration of exposure[^] (w –weeks d- days) | Antibody response | Method of antibody detection | Reference |
|---|---|---|---|---|---|---|---|---|
| **Protozoa** | | | | | | | | |
| *Trichodina murmanica,* Poljansky | Winter flounder *Pseudopleuronectes americanus* Walbaum | + p[2] | | 10-14 | 10 w | nd | | (Barker, et al., 2002) |
| *Amyloodinium ocellatum* | Tomato clown fish *Amphiprion frenatus* Bloch | + | | np | 30 min exposure, 3 d infection, repeated × 4 | + Serum | IF WB | (Cobb, et al., 1998) |
| *Oodinium* spp. | Goldfish *Carassius auratus* L. | | + | 28-30 | np | + Serum + Mucus | E | (Ling, et al., 1993) |
| *Ichthyobodo* spp. | Goldfish | | + | 28-30 | np | + Serum + Mucus | E | (Ling, et al., 1993) |
| *Chilodonella* spp. | Goldfish | | + | 28-30 | np | + Serum + Mucus | E | (Ling, et al., 1993) |

*Table 1.1 continued*

| Ectoparasite | Host | Host resistance+ Exposure | Vaccination | Temperature (°C) | Duration of exposure^ (w –weeks d- days) | Antibody response | Method of antibody detection | Reference |
|---|---|---|---|---|---|---|---|---|
| **Protozoa** | | | | | | | | |
| *Neoparamoeba* spp. | Atlantic salmon *Salmo salar* L. | + p | | 16 | 72 d | nd | | (Bridle, et al., 2005) |
| | | + s | | 14 | 4 w | + Serum[6] | E | (Findlay, et al., 1995) |
| | | + s | | 14 | 4 w | nd | | (Findlay & Munday, 1998) |
| **Monogenea** | | | | | | | | |
| *Gryodactylus pleuronecti* Cone | Winter flounder | + p[2] | | 10-14 | 10 w | nd | | (Barker, et al., 2002) |
| *Discocotlye sagittata* | Rainbow trout *Oncorhynchus mykiss* (Walbaum) | | + | 13 | 4 w | + Serum | E WB | (Rubio-Godoy, et al., 2003b) |
| *Discocotlye sagittata* | Rainbow trout | - p | | np | Sampled fish from 0+, 1+ and 2+ year classes | + Serum | E | (Rubio-Godoy, Sigh, Buchmann & Tinsley, 2003a) |

*Table 1.1 continued*

| Ectoparasite | Host | Host resistance+ Exposure | Vaccination | Temperature (°C) | Duration of exposure^ (w –weeks d- days) | Antibody response | Method of antibody detection | Reference |
|---|---|---|---|---|---|---|---|---|
| **Monogenea** | | | | | | | | |
| *Gyrodactylus salaris* | Atlantic salmon | nd | | 10 | 6 w | - Serum<br>- Mucus | WB | (Buchmann, Madsen & Dalgaard, 2004) |
| *Neobenedenia girellae* Hargis | Japanese flounder *Paralichthys olivaceus* Temminck and Schlegel | nd | | np | 107 d | + Serum<br>+ Mucus | E<br>WB | (Hatanaka, Umeda, Yamashita & Hirazawa, 2005) |
| *Microcotyle sebastis* | Rockfish *Sebastes schlegelii* Hilgendorf | | + | 18 | 1 w exposure, assessed after 7 w | nd | | (Kim, Hwang, Cho & Park, 2000) |
| **Crustacea** | | | | | | | | |
| *Lepeophtheirus salmonis* Kroyer | Atlantic salmon | nd | | N | 104 w F | + Serum | E<br>WB | (Grayson, Jenkins, Wrathmell & Harris, 1991) |

*Table 1.1 continued*

| Ectoparasite | Host | Host resistance+ Exposure | Vaccination | Temperature (°C) | Duration of exposure^ (w – weeks d- days) | Antibody response | Method of antibody detection | Reference |
|---|---|---|---|---|---|---|---|---|
| **Mollusca** | | | | | | | | |
| Larvae of the broken rays mussel *Lampsilis reeveiana* | Largemouth bass *Micropterus salmonides* Lacepide | + s | | 22-23 | 52 w | + Serum | WB | (Dodd, et al., 2006) |
| Larvae of the freshwater mussel *Utterbackia imbecillis* | Bluegill sunfish *Lepomis macrochirus* Rafinesque | nd | | 20-21 | 80 d<br>100 d | + serum<br>+ mucus[1] | E, WB, IF<br>E | (Rogers-Lowery, et al., 2007) |

+ positive, - negative, nd - not determined, np – not provided, p – prolonged, s – secondary, L – laboratory, F – field, E – ELISA, WB – Western blot, IF – immunofluorescence, [+] Resistance in terms of reduced parasitemia or increased survival.
[^] Duration of exposure = total exposure time or time secondary exposure or post-booster vaccination. [1] – titre higher than control at a single point over time. p[2] – experiment conducted with fluctuations in water temperature and salinity. [6] antibodies detected after binding to cultured antigen.

**Vaccination against fish parasites**

Vaccination of fish provides an attractive alternative to chemical or pharmaceutical treatment of fish diseases. For many bacterial and viral fish pathogens, candidate vaccine antigens have been identified by immunisation studies. A serum antibody response can be elicited in fish following immunisation of purified or crude parasite antigen(s) (Table 1.2). For example, channel catfish, *Ictalurus punctatus* Rafinesque, immunised with live or purified *I. multifiliis* antigens develop an antibody response (Wang, Clark, Noe & Dickerson, 2002; Wang & Dickerson, 2002; Swennes, Findly & Dickerson, 2007). Rainbow trout developed a modest serum antibody response after immunisation with cultured *N. pemaquidensis* in Freund's Complete Adjuvant (FCA). Peak antibody responses were observed after 6 weeks following a single immunisation of 1 mg total protein (TP) (Akhlaghi, Munday, Rough & Whittington, 1996) and 4 weeks post booster when two immunisations of 0.01 mg TP were administered (Bryant, Lester & Whittington, 1995) (Table 1.2). However, a disadvantage of performing immunisation studies is the requirement for high quantities of antigen and this, in many cases, is not practical.

Cultured *Neoparamoeba* spp. tested to date are avirulent (Kent, et al., 1988; Howard, et al., 1993; Findlay, 2001; Morrison, et al., 2005; Vincent, et al., 2007a). Furthermore, immunisation of Atlantic salmon with cultured *N. pemaquidensis* did not elicit resistance to AGD (Akhlaghi, et al., 1996; Zilberg & Munday, 2001). Therefore, cultured *Neoparamoeba* spp. antigens are not considered suitable for the assessment of resistance

of Atlantic salmon to AGD or for identifying candidate AGD vaccine antigens. Bath immersion (Morrison & Nowak, 2005) or i.p immunisation (Zilberg & Munday, 2001) of Atlantic salmon with low numbers of wild-type *Neoparamoeba* spp. also failed to confer protection to AGD although low numbers of parasites were administered. The only source of wild-type *Neoparamoeba* spp. is from AGD-affected Atlantic salmon. At the University of Tasmania, Australia, wild-type *Neoparamoeba* spp. are isolated from the gill tissues of AGD-affected Atlantic salmon following the protocol described by Morrison, Crosbie and Nowak, (2004). The average yield of wild-type *Neoparamoeba* spp. is $10^6$ cells (equivalent to around 140 μg TP) from up to seven AGD-affected fish. It is difficult to determine the amount of wild-type *Neoparamoeba* spp. antigen that would be required to elicit an antibody response in Atlantic salmon. A modest antibody response was detected in rainbow trout immunised with 1 mg TP of sonicated *N. pemaquidensis* with Freund's complete adjuvant (Akhlaghi, et al., 1996). Rainbow trout develop a serum antibody response to sea lice, *Lepeophtheirus salmonis* Kroyer, after immunisation with 1.5 mg TP/fish of sea lice antigens in adjuvant (Table 1.2). To obtain 1.5 mg TP from wild-type *Neoparamoeba* spp., approximately $10^7$ wild-type *Neoparamoeba* spp. from an estimated 50-60 AGD-affected Atlantic salmon would be required to immunise a single fish. Immunisation studies with wild-type *Neoparamoeba* spp. to assess an antibody response and/or protection of Atlantic salmon against AGD are impractical due to the time associated with isolating wild-type *Neoparamoeba* spp. and the large numbers of donor fish required.

**Table 1.2.** Conditions applied for the i.p immunisation of fish against parasites. The parasite antigen and total protein concentration, use of adjuvant, antibody activity and the time post-booster (PB) when the antibody response reaches its peak are summarised (where more than one booster is given the time is post-final booster).

| Fish species | Parasite Antigen (Total protein - μg) [parasite number] | Immunisation schedule (weeks) and use of adjuvant | Temperature (°C) | Antibody activity[1] (fold increase[2]) | Peak antibody response (weeks) | Reference |
|---|---|---|---|---|---|---|
| Channel catfish *Ictalurus punctatus* Rafinesque | *Ichthyophthirius multifiliis* Live theronts (12.5) | 0, 19 | np | ImmA (80-2560) | 14 PB | (Swennes, et al., 2007) |
| | *I. multifiliis* i-antigens (10) | 0 + FCA, 2 + FCA | 20-23 | ImmA (2.5-3) | 2 PB | (Wang, et al., 2002) |
| | *I. multifiliis* i-antigens (10) | 0 + FCA, 2 + FIA | 20-23 | ImmA (640) | 5 PB | (Wang & Dickerson, 2002) |
| | *I. multifiliis* Live theronts (20 then 25)[4] | 0, 5 | 20-23 | ImmA (480) | 7 PB | (Wang & Dickerson, 2002) |
| Rainbow trout *Oncorhynchus mykiss* (Walbaum) | *I. multifiliis* Live theronts (2) | 0 | 20 | ImmA (>10) | 14 | (Alishahi & Buchmann, 2006) |

***Table 1.2 continued***

| Fish species | Parasite Antigen (Total protein - µg) [parasite number] | Immunisation schedule (weeks) and use of adjuvant | Temperature (°C) | Antibody activity[1] (fold increase[2]) | Peak antibody response (weeks) | Reference |
|---|---|---|---|---|---|---|
| Rainbow trout | *Lepeophtheirus salmonis* (500) | 0 + FCA, 3 + FCA, 6 + FIA | 12-14 | ELISA (>4) | 4 PB | (Grayson, et al., 1991) |
| | *Neoparamoeba pemaquidensis* (1000) | 0 + FCA | 15 | ELISA (<3) | 6 | (Akhlaghi, et al., 1996) |
| | *Neoparamoeba pemaquidensis (10)* | 0 + FCA, 4 + FCA | 15 | ELISA (4-7.3)[5] | 4 PB | (Bryant, et al., 1995) |
| Japanese flounder *Paralichthys olivaceus* Temminck and Schlegel | *Neobenedenia girellae* Hargis (27-53)[2] | 0 + FCA, 2 + FCA | np | ELISA (>3) | 6 PB | (Hatanaka, et al., 2005) |
| Grouper *Epinephelus coioides* Hamilton | *Cryptocaryon irritans* Brown [30,000] | 0, 2 | np | ELISA (300)[3] | 2 PB | (Luo, Xie, Zhu & Li, 2006) |

[1]antibody activity assessed by immobilisation assay (ImmA) or enzyme linked immunosorbent assay (ELISA). [2]fold increase expressed as the increase in activity compared to the control measured by optical density (ELISA) or cell agglutination (ImmA). [2] fish immunised with cilia antigens 500 µg/kg fish. [3]peak response was highly variable and declined rapidly over time. [4] protein concentration based on protein concentration of theronts described by Alishahi and Buchmann, (2006). [5]data variable and from only 3 fish. FCA: Freund's Complete Adjuvant, FIA: Freund's Incomplete Adjuvant, PB: post-booster, np: detail not provided.

Other research groups are currently undertaking AGD vaccine research. Monoclonal antibodies (MAbs) are being developed to identify antigens specific to wild-type *Neoparamoeba* spp. (Villavedra, Lemke, To, Broady, Wallach & Raison, 2007). Inhibitory action of monoclonal antibodies can be assessed *in vitro* and subsequently the protective qualities of these MAbs can be assessed *in vivo*. For example, passive immunisation of channel catfish with *Ichthyophthirius multifiliis* immobilising monoclonal antibodies confers protection to *I. multifiliis* challenge (Lin, Clark & Dickerson, 1996). A combination of bioinformatics and cDNA expression library immunisation (ELI) represents another approach of AGD vaccine research (Cook, Campbell, Patil, Elliott & Prideaux, 2007). ELI has produced promising results in animal models against a range of pathogens (Talaat & Stemke-Hale, 2005). In addition to the above-mentioned approaches, immune sera can be exploited to identify parasite antigens that are expressed *in vivo*.

## Identifying candidate vaccine antigens

### *Serum antibodies as a tool for identifying candidate vaccine antigens*

Antigen discovery for human pathogens has advanced with increasing development of molecular techniques and where cDNA expression libraries are available, immune sera can be used to screen recombinant proteins. *In vivo* induced antigen technology (IVIAT) utilises serum from infected individuals to screen antigens against expression library antigens from the pathogen of interest (Handfield, Brady, Progulske-Fox & Hillman, 2000). IVIAT has been used to identify vaccine candidate antigens of *Plasmodium falciparum*, the causative agent of *falciparum* malaria (Nixon, Friedman, Knopf, Duffy & Kurtis, 2005);

*Leishmania donovani*, the causative agent of kala azar (visceral leishmaniasis) (Arora, Pal & Mujtaba, 2005) and *Escherichia coli* O157 responsible for a range of potentially fatal human conditions (John, Kudva, Griffin, Dodson, McManus, Krastins, Sarracino, Progulske-Fox, Hillman, Handfield, Tarr & Calderwood, 2005).

Immune sera can also be screened against crude or purified pathogen antigens to identify candidate vaccine antigens. Antibodies in human immune sera binds the Gal/GalNAc inhibitable lectin of *Entamoeba histolytica* Schaudinn, (Petri, Joyce, Broman, Smith, Murphy & Ravdin, 1987; Abd Alla, Jackson, Soong, Mazanec & Ravdin, 2004) and this lectin has shown promise as a vaccine candidate in animal models (Zhang, Cieslak & Stanley, 1994; Soong, Kain, Abd-Alla, Jackson & Ravdin, 1995; Dodson, Lenkowski, Eubanks, Jackson, Napodano, Lyerly, Lockhart, Mann & Petri, 1999).

The discovery of immobilisation antigens of the ciliate *I. multifiliis* is the most notable example of using immune sera for antigen discovery for a fish parasite. Immobilisation of *I. multifiliis* theronts by immune sera was first observed by Hines and Spira (1974). Subsequently, it was shown that antibody binding resulted in parasite immobilisation by binding to ciliary and cell-surface antigens (Clark, Dickerson & Findlay, 1988; Clark & Dickerson, 1997). Immunisation trials have since shown that purified immobilisation antigens (Wang & Dickerson, 2002) or live theronts (Alishahi & Buchmann, 2006; Swennes, et al., 2007) induce serotype-specific antibody-mediated protection to white spot, caused by *I. Multifiliis,* in channel catfish.

In this project, sera from AGD-affected Atlantic salmon were screened against cultured and wild-type *Neoparamoeba* spp. with the aim of identifying candidate vaccine antigens. This approach provides another complementary arm to the AGD vaccine research effort.

***Parasite attachment receptors as candidate vaccine antigens***

Parasite cell-surface lectins are commonly associated with attachment of pathogenic amoebae to host tissues. The binding specificity of the carbohydrate recognition domain (CRD) of the parasite lectin used for attachment to host tissues may be inferred by screening monoclonal antibodies (MAbs), saccharides, host tissues or other compounds for inhibitory activity *in vitro* (Table 1.3). Once identified, the regions associated with cell attachment may be produced by recombinant technology and assessed *in vivo* as candidate vaccine antigens. Recombinant proteins representing regions of the CRD of the Gal/GalNAc inhibitable lectin of *E. histolytica* confer protection against amoebic liver abscess in gerbils (Gaucher & Chadee, 2003). In addition, collagen-binding proteins (CBP) are also implicated in *E. histolytica* attachment. Immunisation of hamsters with recombinant CBP provides protection against liver abscess (Jimenez-Delgadillo, Chaudhuri, Baylon-Pacheco, Lopez-Monteon, Talamas-Rohana & Rosales-Encina, 2004).

There have been very few studies that compare surface antigens of closely related amoebae. However, antigenic similarities in closely related parasites that affect humans, cattle and avian species have been studied, predominately on a molecular level.

Antigenic similarities can be retained throughout evolution. For example,different species of amoebae can have similar antigenic properties, particularly if they are closely related as is seen with *Hartmannella vermiformis* and *Entamoeba histolytica* (Venkataraman, Haack, Bondada & Kwaik, 1997). Mechanisms associated with attachment of wild-type *Neoparamoeba* spp. are unknown, cell-surface receptors may represent novel candidate vaccine antigen(s) for an AGD vaccine. In this project, the effect of mucus and a range of saccharides on the ability of wild-type *Neoparamoeba* spp. to colonise the gill tissues of Atlantic salmon *in vivo* were assessed.

**Table 1.3.** The attachment of pathogenic amoebae can be inhibited *in vitro* by saccharides, host tissues or antibodies. Inhibition of attachment of a range of pathogenic amoebae to a substrate or cell line by saccharides or host target tissues are summarised.

| Parasite | Substrate | Inhibitor | Reference |
|---|---|---|---|
| *Acanthamoeba castellanii* | Rabbit corneal epithelial cells | Mannose | (Yang, Cao & Panjwani, 1997; Cao, Jefferson & Panjwani, 1998) |
| *Acanthamoeba* spp. | Human brain endothelial cells | Mannose | (Alsam, Kim, Stins, Rivas, Sissons & Khan, 2003) |
| *Acanthamoeba polyphaga* | Madin-Darby canine kidney cells<br>Contact lens | Human sIgA | (Campos-Rodriguez, Oliver-Aguillon, Vega-Perez, Jarillo-Luna, Hernandez-Martinez, Rojas-Hernandez, Rodriguez-Monroy, Rivera-Aguilar & Gonzalez-Robles, 2004) |
| *Entamoeba histolytica* | CHO cells | MAbs | (Ravdin, Petri, Murphy & Smith, 1986) |
| | CHO cells | Mucins | (Chadee, Petri, Innes & Ravdin, 1987) |
| | CHO cells | MAbs | (Abd Alla, et al., 2004) |
| *Giardia lamblia* | 96-well plate | Mucin | (Roskens & Erlandsen, 2002) |
| | Caco-2 cells | Mannose | (Katelaris, Naeem & Farthing, 1995) |
| *Naegleria fowleri* | Collagen 1 | Human sIgA<br>Rabbit IgG | (Shibayama, Serrano-Luna, Rojas-Hernandez, Campos-Rodriguez & Tsutsumi, 2003) |

For the development of an alternative to fresh water bathing for AGD of Atlantic salmon, such as vaccination, it is essential to first determine if Atlantic salmon are able to develop resistance to wild-type *Neoparamoeba* spp.. Secondly, suitable candidate vaccine antigens for an anti-AGD vaccine must be identified before the subsequent assessment of their protective qualities can be made. Therefore the specific aims of this project are as follows:

- Assess the ability of Atlantic salmon to develop resistance to AGD.
- Assess the development of a serum antibody response in Atlantic salmon to wild-type *Neoparamoeba* spp..
- Identify potential AGD vaccine candidate antigen(s) by screening sera from AGD-affected Atlantic salmon against cultured and wild-type *Neoparamoeba* spp. antigens.
- Assess potential mechanisms of attachment employed by *Neoparamoeba* spp. to colonise Atlantic salmon gill tissues by screening mucus and a range of saccharides *in vivo*.

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# Chapter 2

# Amoebic gill disease (AGD) affected Atlantic salmon (*Salmo salar* L.) are resistant to subsequent AGD challenge

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**Abstract**

There is inconsistent evidence of resistance of Atlantic salmon, *Salmo salar* L., to amoebic gill disease (AGD). Here, evidence is presented that Atlantic salmon exposed and subsequently challenged with AGD are more resistant in terms of reduced mortality than naïve control fish. Seventy three percent of Atlantic salmon previously exposed to AGD survived to day 35 post-challenge compared to 26% exposed to *Neoparamoeba* spp. for the first time, yet the gill pathology of surviving naïve control or previously exposed fish was not significantly different. Anti-*Neoparamoeba* spp. antibodies that were detectable in serum of 50% of surviving Atlantic salmon previously exposed to AGD. However, anti-*Neoparamoeba* spp. antibodies were not detectable in cutaneous mucus of resistant fish. Reduced mortality of Atlantic salmon after secondary *Neoparamoeba* spp. infection and detection of specific serum antibodies provides support for the development of a vaccine for AGD.

## Introduction

Vaccines developed against a range of bacterial fish pathogens have provided the aquaculture industry with relief from the use of antibiotics. Perhaps the most notable instance was the drastic reduction in antibiotic use in Norway that followed the introduction of vaccines against vibriosis and furunculosis during the late 1980s (Sommerset, Krossoy, Biering & Frost, 2005). This resulted in a significant increase in the production of farmed fish. However, the development of vaccines against parasitic pathogens of fish has proven more difficult. There is an increasing amount of evidence for the role of adaptive immunity in protection against fish parasites and this provides encouragement for future development of anti-parasitic vaccines. For example, resistance to the monogenean *Discocotyle sagittata* (Rubio-Godoy, et al., 2003a), dinoflagellate *Amyloodinium ocellatum* (Cobb, et al., 1998) and haemoflagellate *Cryptobia salmositica* (Chin & Woo, 2005) has been associated with specific serum antibody. In addition the response of channel catfish to infection with the protozoan *I. multifiliis* has been widely studied and there is strong support for the protective role of both mucosal and systemic antibody (Wang & Dickerson, 2002; Xu & Klesius, 2002). Further, antibodies that bind immobilization antigens mediate resistance to *I. multifiliis* infection (Clark, Lin & Dickerson, 1996; Lin, et al., 1996).

The aetiological agent of amoebic gill disease (AGD) of Atlantic salmon is *Neoparamoeba* spp. (Adams & Nowak, 2004a; Dyková, et al., 2005a). In Tasmania, Australia, AGD is most prevalent during summer months and is associated with increased water temperature and 35‰ salinity (Clark & Nowak, 1999; Adams & Nowak, 2003). The characteristic gross sign of *Neoparamoeba*

spp. infection is white raised mucus patches on infected gill surfaces. Histologically, infection with *Neoparamoeba* spp. results in hyperplasia of epithelial cells leading to fusion of secondary gill lamellae (Roubal, et al., 1989; Zilberg, Findlay, Girling & Munday, 2000; Adams & Nowak, 2001). To date, the only effective treatment for AGD is freshwater bathing and developing an alternative treatment such as a vaccine is a priority for Tasmanian salmon growers.

Resistance of Atlantic salmon to AGD after secondary exposure has been previously reported on the basis of gill pathology (Findlay, et al., 1995; Findlay & Munday, 1998) and more recently, enhanced survival of a sub-population of Atlantic salmon to AGD infection has been documented in association with a marked reduction in gill pathology (Bridle, et al., 2005). While there is evidence that Atlantic salmon are able to develop resistance to AGD, conflicting results also exist. Resistance to AGD challenge was not seen after Atlantic salmon were previously exposed to *Neoparamoeba* spp. (Gross, et al., 2004b). However, results from this study may have been influenced by mortalities that were recorded prior to AGD challenge and further by the presence of an ongoing AGD infection in one treatment group. Due to inconsistent reports, the need remains for further study to identify if Atlantic salmon can develop resistance to AGD.

Identifying a protective antibody response against wild-type *Neoparamoeba* spp. is central to the development of a successful vaccine treatment for AGD. As *Neoparamoeba* spp. are found predominately in association with the surface of gill lesions, the role of antibodies in resistance to AGD had been largely dismissed. However, *Neoparamoeba* spp. can be found entrapped within

interlamellar vesicles in association with inflammatory cells (Adams & Nowak, 2001). The subsequent processing of entrapped antigen may be facilitated by MHC $II^+$ cells that are present in gill lesions of AGD-affected Atlantic salmon (Morrison, et al., 2006) resulting in production of specific antibody. While detectable, yet modest systemic antibody has been previously reported in Atlantic salmon after contracting AGD (Akhlaghi, et al., 1996; Gross, Carson & Nowak, 2004a), these antibodies were detected after binding to cultured amoebae antigens. Cultured gill-derived *Neoparamoeba* spp. are avirulent (Morrison, et al., 2005) and antibodies that bind cultured amoebae may not be relevant *in vivo*. It is essential that the antibody response of Atlantic salmon to wild-type *Neoparamoeba* spp. be assessed if antigens associated with virulence are to be discovered. The present study investigates the effect of *Neoparamoeba* spp. infection on the development of a detectable systemic antibody response and survival of Atlantic salmon. Results presented here show that Atlantic salmon are able to develop resistance, in terms of reduced mortality, to AGD and systemic antibody is associated with some AGD-resistant fish.

## Materials and methods

*Fish and experimental conditions*

Atlantic salmon with an average weight of 95.2 ± 4.5g were obtained from the Saltas hatchery, Wayatinah, Tasmania. These fish had only been maintained in fresh water and therefore had not been exposed to *Neoparamoeba* spp.. Fish were held in 3000 L temperature controlled recirculating systems, each with an individual protein skimmer and biofilter. Prior to experimental procedures, fish were acclimated to 35‰ salinity by multiple sea water exchanges over a 10 d period. Sea water was filtered to 1 μm. During experimental procedures, tanks were subjected to twice-weekly water exchange of approximately 30% volume.

Fish were fed once daily to satiation using a commercial 3 mm pellet (Skretting, Tasmania).

This study involved two independent trials. The first exposed Atlantic salmon to *Neoparamoeba* spp. infection at 12°C for a period of 4 weeks and was performed to obtain serum and mucus samples. The second trial was conducted to assess resistance of Atlantic salmon to AGD challenge. The initial infection period was conducted at 12°C to maintain the infection for 4 weeks at a non-lethal level. This was to ensure that fish in trial 2 could proceed to challenge and that the survival of these fish was not influenced by a heavy *Neoparamoeba* spp. infection when exposed to challenge conditions.

*Trial 1- Induction of amoebic gill disease*

Two tanks of 30 Atlantic salmon were acclimated from 12°C to 16°C by increasing the water temperature by 1°C per day. To establish infection, gill-derived amoebae (Morrison, et al., 2004) were introduced to one tank at 500 cells/L. After 7 d at 16°C, the water temperature of both tanks was reduced by 1°C per day to 12°C and fish were monitored for a period of 4 weeks. These fish were terminally sampled after the 4 week infection period.

*Trial 2- Induction of amoebic gill disease and challenge*

Two tanks of 40 fish were subjected to the same regime as trial 1. After the initial 4 week infection period at 12°C, 10 fish from each tank were randomly sampled following the sampling protocol outlined below. The remaining 30 fish from each tank were transferred into a freshwater bath for 24 h at 14°C. After

bathing, one group was tagged by sub-dermal injection with Alcian blue (Sigma-Aldrich) and all 60 fish (30 fish previously exposed and 30 naïve fish) were returned to the system that was previously inoculated with *Neoparamoeba* spp.. No further amoebae were added and the temperature was adjusted to 15°C then to 16°C the following day. The currently accepted criterion used to assess the efficacy of vaccines administered by immersion is that the relative percent survival of treated fish is >60% and the control mortality is 60% or greater, 21 days after onset of disease (Midtlyng, 2005). This criterion was adopted to identify if Atlantic salmon previously exposed to *Neoparamoeba* spp. displayed resistance when subsequently challenged with AGD.

**Sampling and assessment of gill pathology**

Fish were killed by overdose of Aqui-S (Aqui-S NZ Ltd, Lower Hutt, New Zealand). Cutaneous mucus was collected from each fish using a cotton swab and placed into an Eppendorf tube containing 400 µL PBS and 100 µL anti-protease cocktail (Sigma-Aldrich). The tubes were vortexed to elute the mucus and excess liquid was removed from the swab by pressing against the side of the tube. Samples were centrifuged at 16 000× *g* for 15 min to separate mucus from particulate contamination and the supernatant was removed and stored at –20°C. Blood was taken from the caudal vein, allowed to clot overnight at 4°C, centrifuged at 1000× *g* for 10 min and serum was removed and stored at -20°C. The entire gill basket was excised and placed in seawater Davidson's fixative (SWD). Gills were transferred to 70% ethanol after 24 h. To view gross pathology, the second left gill arch was photographed (Olympus C5050, Tokyo, Japan) and then the same gill arch was processed and embedded following

routine histological protocols. Sections (5 μm) were stained with hematoxylin and eosin (H and E). The proportion of filaments affected by *Neoparamoeba* spp. was assessed by light microscopy at 400× magnification. Filaments were counted when the central venous sinus was visible in at least two-thirds of the filament length (Adams & Nowak, 2003).

**Cultured and wild-type amoebae**

Wild-type amoebae were isolated as described by Morrison et al., (2004) from *Neoparamoeba* spp. infected Atlantic salmon housed at the University of Tasmania aquaculture research centre. To discriminate between antibody binding to cultured and wild-type *Neoparamoeba* spp. antigens, two previously characterised clonal strains of cultured *Neoparamoeba* spp. were included for Western blot analysis. These were *Neoparamoeba pemaquidensis* (NP251002) (Morrison, et al., 2005) isolated from AGD-affected Atlantic salmon, and *Neoparamoeba branchiphila* (SEDMH1) (Dyková, et al., 2005a) isolated from marine sediment. Amoebae were maintained on seawater malt yeast agar; 75% (v/v) coarse filtered seawater (35‰), 25% (v/v) distilled water, 0.01% (w/v) Malt, 0.01% (w/v) yeast (Oxoid, Hampshire, England), 2% (w/v) Bacto agar (Becton, Dickson and Co., USA). Cells were harvested by washing from the agar with sterile sea water using a transfer pipette. Wild-type and cultured cells were concentrated by centrifugation at 500× *g* for 5 min and enumerated by hemacytometer. Amoebae were washed twice with PBS and the cell pellet was stored at -80° C until use.

**Detection of anti-*Neoparamoeba* spp. antibodies in serum and mucus**

*Positive and negative control serum*

Serum was obtained from an Atlantic salmon that had been exposed to *Neoparamoeba* spp. and displayed overt signs of resistance. This fish presented a low level of gross gill pathology and prolonged survival in challenge conditions. Serum antibodies bound to wild-type *Neoparamoeba* spp. in an indirect enzyme linked immunosorbent assay (ELISA) and this serum was further characterised and used as a positive control in subsequent Western blot and ELISA assays. Negative control sera was pooled from 5 fish maintained in fresh water and therefore these fish were naïve to AGD.

*SDS-PAGE and Western blot*

To identify the binding activity and specificity of serum antibody against cultured and wild-type amoebae antigens, all serum samples taken (at the end of trial 1, prior to freshwater bathing in trial 2 and at the termination of trial 2) were first processed by Western blot. Initially, pools of serum from 5 fish were screened and serum from pools returning a positive result were subsequently screened individually. Amoebae antigens were reduced in buffer containing β-mercaptoethanol by boiling for 10 min, separated through 6% polyacrylamide gels with 4 x $10^4$ cell equivalents were loaded in each lane (5.5 μg total protein per lane). Antigens were transferred to nitrocellulose membrane (Hybond-C extra, Amersham Biosciences, UK) using a semi-dry transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA). Membranes were blocked in casein solution (Vector, Burlingame, CA, USA). Blocking and antibody incubation steps were for 30 mins and in between incubation steps, membranes were washed 3 x 4 min with tris-buffered saline (TBS, pH 7.2). Atlantic salmon serum was applied at 1:100 (pooled) and 1:500 (individual). Bound antibodies were detected with rabbit anti-salmon IgM at 1:5000 followed by alkaline phosphatase

(AP)-conjugated sheep anti-rabbit IgG (Chemicon, Australia) at 1:5000. For analysis of mucus, antigen concentration was increased to 8 x $10^4$ cells per lane and mucus supernatant was diluted 1:1 in casein solution. Each sample of mucus was assessed for the presence of mucosal anti-wild-type *Neoparamoeba* spp. Bound antibodies were detected with rabbit anti-salmon IgM at 1:500 and AP-conjugated sheep anti-rabbit IgG 1:2000. Mucus collected from 5 Atlantic salmon held only in fresh water was pooled and used as a negative control. Following the final antibody incubation, membranes were washed 3 × in TBS and then in 0.1M tris (pH 9.5) for 5 min. The binding of polyclonal rabbit anti-salmon IgM to Atlantic salmon IgM was initially assessed by western blotting. Normal Atlantic salmon serum and mannan-binding protein (MBP)-purified Atlantic salmon serum IgM were separated through a 12% gel. Proteins were transferred and the membrane was blocked as outlined above. The membrane was probed with polyclonal rabbit anti-salmon IgM and bound antibodies detected with AP-conjugated sheep anti-rabbit IgG as outlined above. All incubation and wash steps were conducted at 20°C. Western blots were developed by enhanced chemiluminescence (ECL) using DuoLuX (Vector), Kodak BioMax Light Film and Kodak GBX developing and fixing reagents (Sigma, Castle Hills, NSW, Australia) following the manufacturer's instructions.

*Enzyme-linked immunosorbent assay (ELISA)*

The activity of anti-*Neoparamoeba* spp. antibodies was determined by an ELISA. Wild-type *Neoparamoeba* spp. suspended in PBS were sonicated then centrifuged for 10 min at 16 000× *g* and the supernatant stored at –20°C. Protein concentration of the sonicated amoebae was determined by a colorimetric assay (Pierce, Rockford, USA). Optimal conditions for ELISA were determined empirically. Briefly, 96-well flat bottom plates (Sarstdet, Australia) were coated with 50 µL sonicated wild-type *Neoparamoeba* spp. (0.24 µg total protein/well) in coating buffer (50 mM $NaHCO_3$, pH 9.5) at 4°C overnight. Antigen was discarded and wells were blocked for 30 min at 37°C with 0.3 % casein-PBS (Sigma). All serum samples were serially diluted in 0.3 % casein-PBS in duplicate from 1:100 to 1:3200, (50 µL/well) and plates were incubated for 1 h at 20°C. Bound antigen was detected with polyclonal rabbit anti-salmon IgM at 1:500 and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG at 1:1000 for 30 min at 37°C. The reaction was developed with 50 µL o-phenylenediamine (OPD) (Sigma) and stopped with an equal volume of 3M HCl. Positive and negative control serum was titrated from 1:100 to 1:3200 on each plate in duplicate.

*Immunocytochemistry and flow cytometry*

Wild-type amoebae were fixed in seawater Davidson's fixative (SWD) for 1 h at 20°C and washed by four cycles of re-suspending cells in PBS and concentrating cells by centrifugation at 500 × *g* for 5 min. Amoebae were placed in wells of 96-well U-bottomed microplates (Sarstdet) and blocked in 0.1% BSA-PBS for 30

min at 4°C. Cells were probed with normal Atlantic salmon serum and serum that contained anti-*Neoparamoeba* spp. antibodies. Cells were incubated with salmon serum at 1:10 and bound antibodies were detected with rabbit anti-salmon IgM at 1:100 and FITC-conjugated sheep anti-rabbit IgG (Chemicon, Australia) at 1:50. Cells were washed 3× in PBS following each antibody incubation step and a sub-sample of cells were photographed (Leica DC300F, Leica Microsystems, Wetzlar, Germany) using light and fluorescent microscopy. Using the remaining probed cells, the proportion of wild-type *Neoparamoeba* spp. expressing epitope(s) to which the salmon anti-*Neoparamoeba* spp. antibodies bound was quantified by flow cytometry (Coulter Epics, Beckman Coulter, USA). Ten thousand cells were assessed per treatment and data were analysed using WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, California, USA).

**Data analysis**

Survival data were analysed by Kaplan Meyer survival analysis with differences in survival between treatment groups determined using the log-rank test (MedCalc). Differences in gill pathology between groups were assessed by t-test. Data were initially tested for homogeneity using Levene's test (SPSS) and differences were considered significant at $P < 0.05$.

**Results**

*Trial 1- Induction of amoebic gill disease*

No mortalities occurred in the *Neoparamoeba* spp. inoculated or control systems during the 4 week infection period. *Neoparamoeba* spp. were not detected on gills of Atlantic salmon from the control tank.

*Trial 2 - Induction of amoebic gill disease and subsequent AGD challenge*

During the initial 4 week infection period of the second trial there were no mortalities recorded in either the inoculated or control tank. Prior to freshwater bathing, gill histology from a sub-population of 10 fish from the inoculated group confirmed AGD. *Neoparamoeba* spp. were not found on gills of Atlantic salmon from the naïve control group. No mortalities occurred during the 24 h freshwater bath treatment. After induction of challenge conditions, morbidity resulting from AGD occurred in the naïve control group after 23 d and after 26 d in the group previously exposed to *Neoparamoeba* spp.. Trial 2 was terminated at day 36 post-challenge by which time 73% of the naïve control group had succumbed to AGD. Cumulative morbidity of naïve fish was significantly greater ($P = 0.001$) than that of Atlantic salmon experiencing secondary exposure to *Neoparamoeba* spp. (Fig. 2.1). Gross and histological observation of gills of surviving fish from the naïve control ($n = 8$) and previously exposed ($n = 22$) groups was consistent with AGD infection (Fig. 2.2) and there was no significant difference ($P = 0.182$) in the proportion of AGD-affected gill filaments between groups (Fig. 2.3).

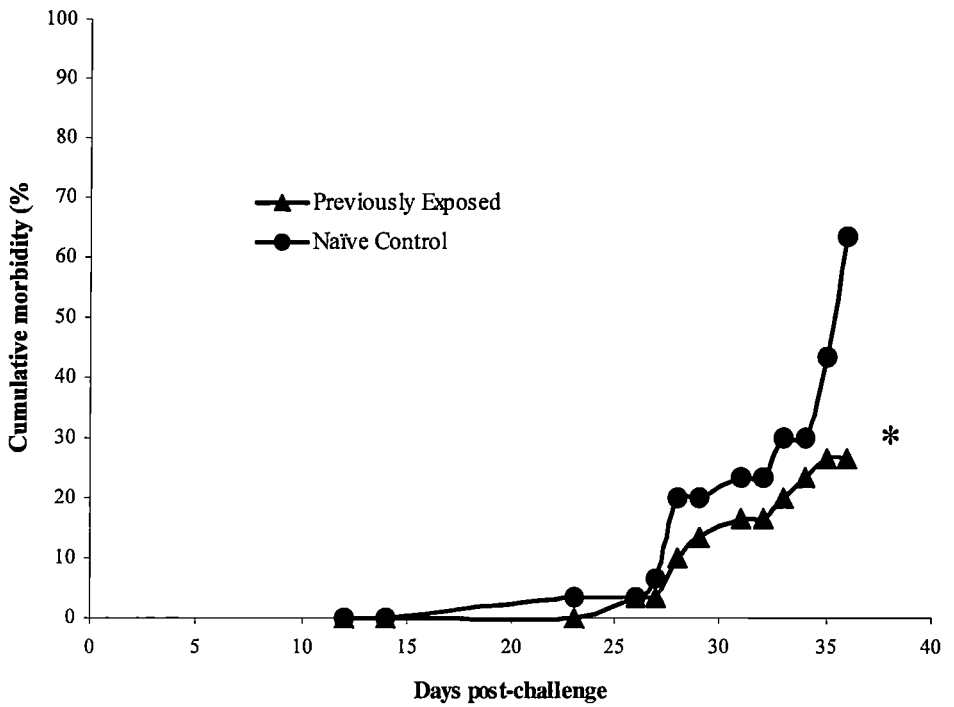
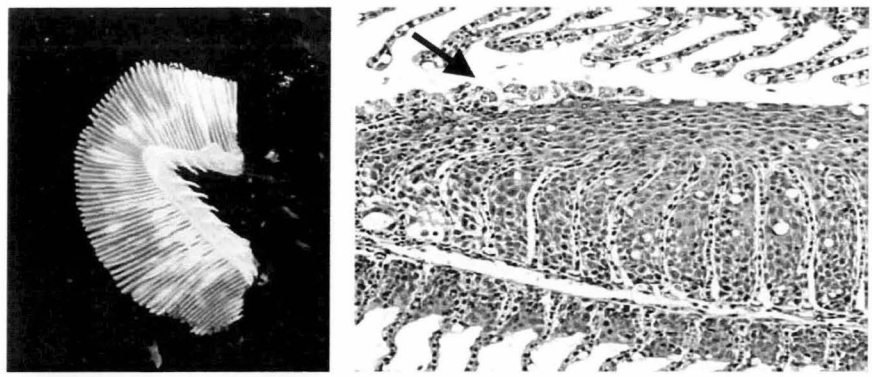
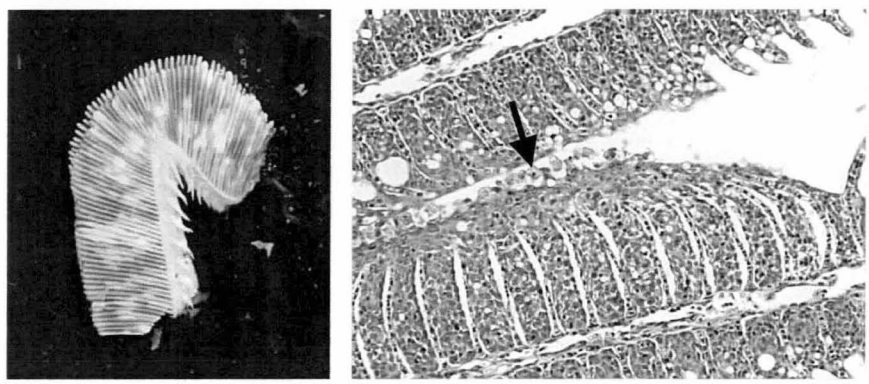



**Figure 2.1.** Atlantic salmon previously infected with *Neoparamoeba* spp. show reduced mortality to a subsequent AGD challenge. Previously exposed fish (n = 30) were inoculated with *Neoparamoeba* spp. at 500 cells/L for 4 weeks while naïve control fish (n = 30) were maintained in seawater. Both groups were bathed in fresh water for 24 h and challenged in the previously inoculated system with no further *Neoparamoeba* spp. added. * P = 0.001.



**Naïve control**



**Previously exposed**

**Figure 2.2.** Atlantic salmon previously infected with *Neoparamoeba* spp. show similar gross and histopathology as naïve control fish after AGD challenge. Gross (left) and histological (right) pathology associated with naïve control and previously exposed Atlantic salmon following *Neoparamoeba* spp. challenge. Pathology is representative of all surviving fish from the respective groups. Histological sections (400x magnification) show the central venous sinus (cvs) and *Neoparamoeba* spp. trophozoites in association with lesion margins (arrows).

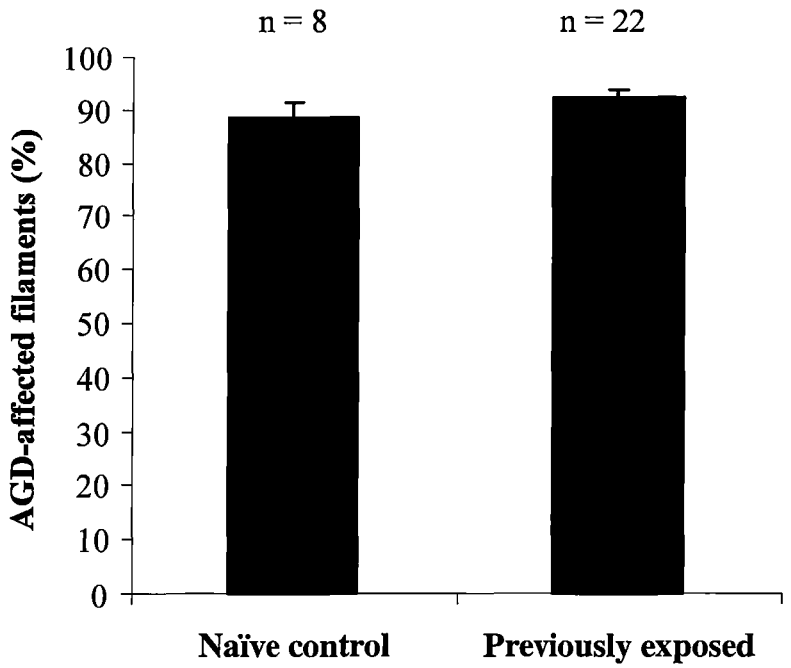



**Figure 2.3.** Previous exposure to *Neoparamoeba* spp. did not influence the proportion of gill filaments affected after subsequent AGD challenge. Analysis of gill histology of surviving fish from the naïve control group (n = 8) and previously exposed group (n = 22) showed similar levels of pathology. Error bars represent SEM.

*Detection of anti-Neoparamoeba spp. antibodies by Western blot and ELISA.*

Serum obtained from a putatively resistant Atlantic salmon that had been exposed to AGD for greater than 3 months contained anti-*Neoparamoeba* spp. antibodies but these antibodies did not bind to cultured amoebae antigens. Normal serum did not contain antibodies reactive with wild-type or cultured antigens (Fig. 2.4). Optical density determined by ELISA of the positive and negative control serum at a dilution of 1:100 was 0.66 ± 0.02 and 0.19 ± 0.02 respectively (Fig. 2.5).

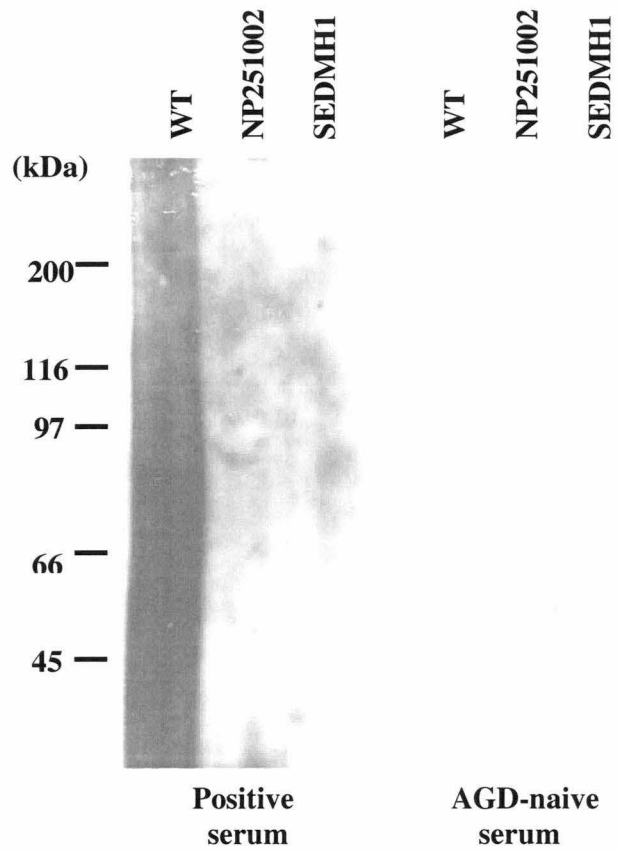



**Figure 2.4.** Anti-*Neoparamoeba* spp. antibodies present in the positive control serum bind specifically to wild-type *Neoparamoeba* spp. (WT). Antibodies do not bind to cultured *Neoparamoeba pemaquidensis* (NP251002) or *Neoparamoeba branchiphila* (SEDMH1). Antigens were reduced in sample buffer containing β-mercaptoethanol, separated through a 6% polyacrylamide gel and each lane was loaded with 4 x $10^4$ cell equivalents. Antigens were transferred to nitrocellulose and probed with positive salmon serum (left) and AGD-naive salmon serum (right). Bound antibodies were detected with rabbit anti-salmon IgM, AP-conjugated sheep anti rabbit IgG and ECL. Positive control serum was obtained from an Atlantic salmon that survived AGD for approximately 3 months. AGD-naïve serum was pooled from 5 fish held in fresh water.

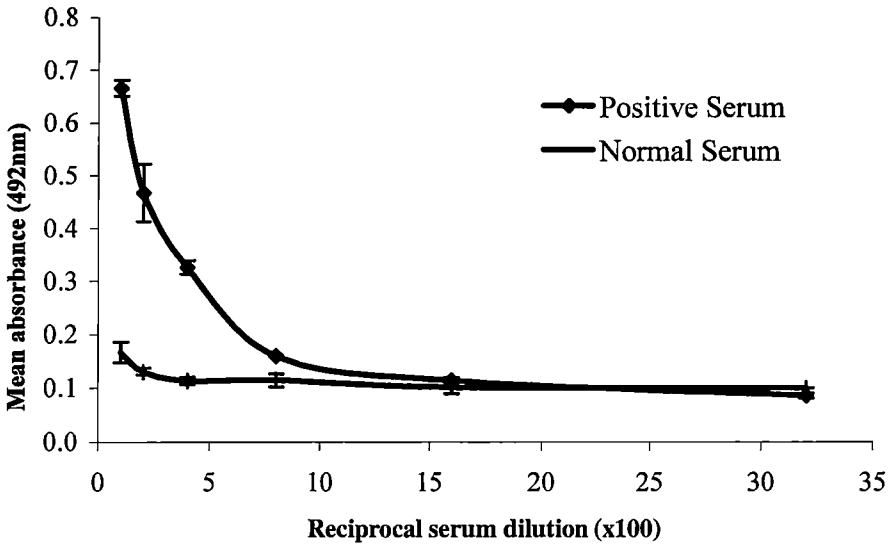



**Figure 2.5.** Positive control serum produces an optical density substantially higher than normal Atlantic salmon serum in ELISA at a dilution of 1:100. ELISA plates were coated with sonicated wild type antigen (0.24 μg total protein/well). Positive and normal serum was titrated from 1:100 to 1:3200 in duplicate. Bound antibody was detected with rabbit anti-salmon IgM, HRP-conjugated goat anti rabbit IgG and OPD. Titration curves represent the mean ± standard error optical density at 492nm. Positive control serum was obtained from an Atlantic salmon that survived AGD for approximately 3 months. Normal sera was pooled from 5 fish held in fresh water and therefore AGD-naïve.

Western blot analysis of serum pooled from fish at the end of the 4 week infection period of trial 1 was negative with no antibody binding to wild-type or cultured *Neoparamoeba* spp. Similarly, in trial 2 anti-*Neoparamoeba* spp. antibodies were not detected by Western blot in pooled serum taken from the 10 infected Atlantic salmon after the initial 4 week infection period. At the end of

trial 2 the serum from all surviving fish (naïve control n = 8, and previously exposed n = 22) was analysed by Western blot. No anti-*Neoparamoeba* spp. antibodies were detected in pooled serum of naïve control Atlantic salmon. However, all serum pools from previously exposed fish were positive for anti-wild-type *Neoparamoeba* spp. antibodies. Western blot analysis of individual samples from these positive pools identified antibodies specific to wild-type amoebae in the serum of 11 of the 22 (50%) surviving fish that had been previously exposed to AGD. The binding profile of all positive samples was consistent, producing an intense smear between 45 and > 200 kDa (Fig. 2.6). This serum was also tested by ELISA but no antibody activity was detected. The average optical density in ELISA of the Western blot positive samples at 1:100 was 0.20 ± 0.01, while the negative control reading was 0.19 ± 0.02.

*Immunocytochemistry and flow cytometry*

Wild-type *Neoparamoeba* spp. were fixed prior to incubation of antibodies to avoid the potential false negative results produced by pinocytosis of antibodies prior to detection. Epi-fluorescent microscopy identified that anti-*Neoparamoeba* spp. antibodies present in a pooled sample of the sero-positive Atlantic salmon serum bound cell-surface antigen(s) of wild-type *Neoparamoeba* spp.. Flow cytometry quantified binding to cell-surface antigen(s) with 85% of the analysed events producing fluorescence intensity an order of magnitude greater than that of the normal serum control (Fig. 2.7).

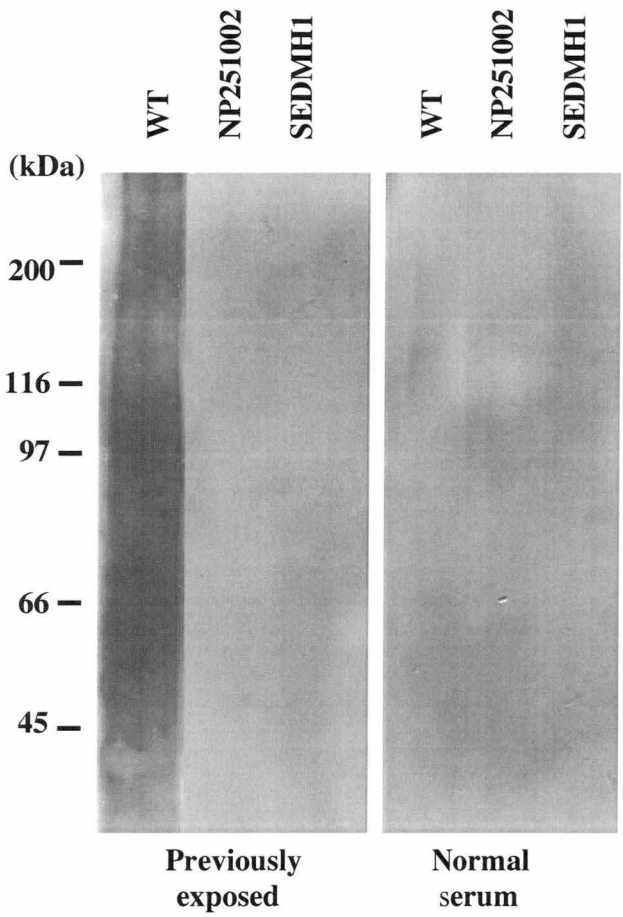



**Figure 2.6.** Serum from an Atlantic salmon previously exposed to *Neoparamoeba* spp. contains antibodies specific to wild-type *Neoparamoeba* spp. (WT). Binding profile of salmon anti-NP serum is representative of 50% of surviving fish that had been exposed to *Neoparamoeba* spp. twice. Antibodies in previously exposed salmon serum do not bind cultured antigens of *Neoparamoeba pemaquidensis* (NP251002) or *Neoparamoeba branchiphila* (SEDMH1). Antigens were reduced in sample buffer containing β-mercaptoethanol. Each lane was loaded with 4 x $10^4$ cell equivalents and antigens were separated through a 6% polyacrylamide gel. Antigens were transferred to nitrocellulose and probed with serum from Atlantic salmon exposed twice to *Neoparamoeba* spp. (left) and normal Atlantic salmon serum (right). Bound antibodies were detected with rabbit anti-salmon IgM, AP-conjugated sheep anti rabbi IgG and ECL. Normal serum was pooled from 5 fish held in fresh water and therefore AGD-naïve.

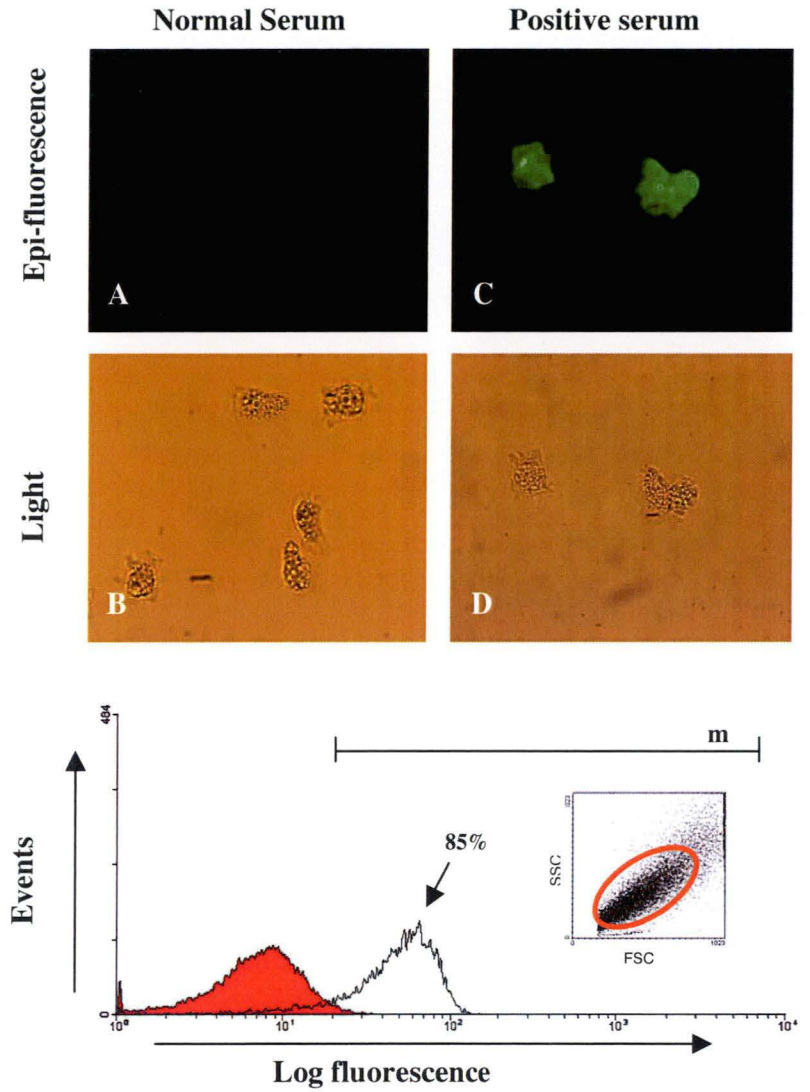



**Figure 2.7.** Anti-*Neoparamoeba* spp. antibodies in pooled positive serum bind cell-surface epitope(s) of wild-type *Neoparamoeba* spp. producing fluorescence intensity significantly higher than that of the normal serum control. Wild–type *Neoparamoeba* spp. were fixed and probed with normal salmon serum (A and C) or pooled positive serum (B and D) at 1:10. Bound antibodies were detected with rabbit anti-salmon IgM at 1:100 and FITC-conjugated sheep anti-rabbit IgG at 1:50. Normal salmon serum was pooled from 5 fish maintained in fresh water and was therefore from AGD-naïve fish. The shaded area of the histogram represents cells probed with normal salmon serum. Cells were photographed under epi-fluorescence and light microscopy. Fluorescence intensity of probed cells was analysed by flow cytometry reading a total of $10^4$ events. The gated region analysed is shown in the dot plot (inset). Flow cytometry data were analysed and presented using WinMDI software.

Western blot analysis of individual cutaneous mucus samples collected from Atlantic salmon showing resistance to challenge did not identify any anti-*Neoparamoeba* spp. antibodies. In general, antibody levels detected in mucus are substantially lower than levels detected in corresponding serum. Given that anti-*Neoparamoeba* spp. antibodies were not detected in mucus from AGD-resistant fish no further analysis of cutaneous mucus was conducted. Rabbit anti-salmon IgM bound crude and purified Atlantic salmon serum IgM (Fig. 2.8). Binding of rabbit anti-salmon IgM to a band at around 70 kDa in cutaneous mucus samples corresponded with the band produced against MBP purified mucosal IgM (Fig. 2.9). This demonstrated that IgM in cutaneous mucus samples had not degraded, that rabbit anti-salmon IgM was able to detect mucosal IgM of Atlantic salmon and the assay was sensitive enough to detect total mucus Ig.

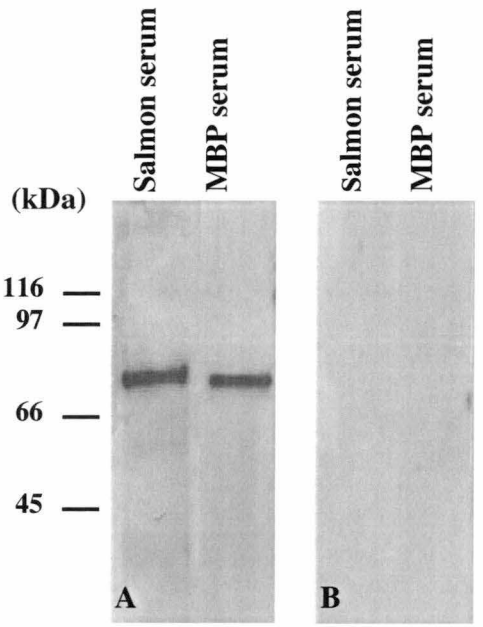



**Figure 2.8.** Polyclonal rabbit anti-salmon IgM binds crude and mannan binding protein (MBP)-purified Atlantic salmon serum IgM. Antigens were reduced in sample buffer containing β-mercaptoethanol, separated through a 12% polyacrylamide gel and transferred to nitrocellulose membrane. Membranes were probed with polyclonal rabbit anti-salmon IgM (A) or normal rabbit serum (B). Bound antibodies were detected with AP-conjugated sheep anti-rabbit IgG and ECL.

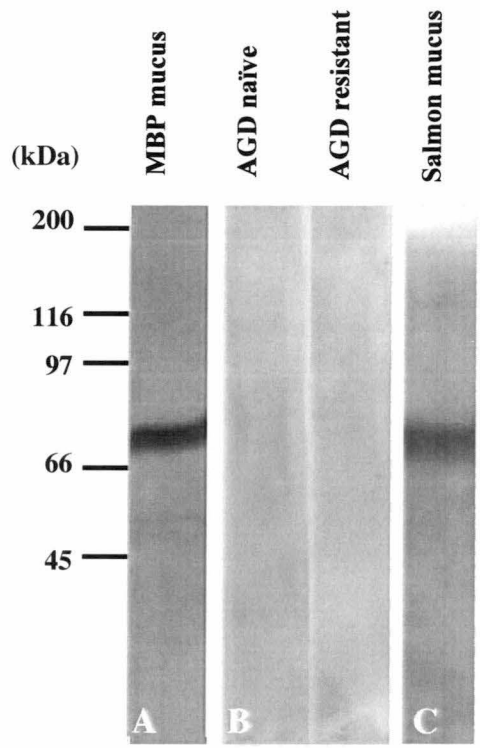



**Figure 2.9.** Polyclonal rabbit anti-salmon IgM binds mannan binding protein (MBP) purified mucosal IgM from Atlantic salmon however anti-*Neoparamoeba* spp. antibodies were not detected in cutaneous mucus of AGD resistant fish. (A) Binding of rabbit anti-salmon IgM to MBP purified mucosal IgM from Atlantic salmon. (B) Anti-*Neoparamoeba* spp. antibody was not detected in cutaneous mucus collected from AGD naïve or previously exposed Atlantic salmon. (C) Detection of total IgM in cutaneous mucus (pooled 4 samples) collected from Atlantic salmon after AGD challenge. Antigens were reduced in sample buffer containing β-mercaptoethanol, separated through a 6% polyacrylamide gel and transferred to nitrocellulose membrane. (A) MBP purified Atlantic salmon mucosal IgM (B) wild-type *Neoparamoeba* spp. ($8 \times 10^4$ cell equivalents per lane) and (C) cutaneous mucus collected from Atlantic salmon with demonstrated systemic antibody. Membrane strips A and C were probed with polyclonal rabbit anti-salmon IgM and bound antibodies detected with AP-conjugated sheep anti-rabbit IgG and ECL. Membrane B was probed with cutaneous mucus from Atlantic salmon that were not exposed (AGD naïve) and Atlantic salmon that were previously exposed to *Neoparamoeba* spp. and showed increased resistance to subsequent AGD challenge (AGD resistant), bound antibodies detected with AP-conjugated sheep anti-rabbit IgG and ECL. AGD naïve mucus was pooled from 5 fish held in fresh water and therefore naïve to AGD.

**Discussion**

This study has shown that primary infection of Atlantic salmon with *Neoparamoeba* spp. enhances protection against subsequent AGD challenge in association with systemic antibodies. The survival of fish that were previously exposed to *Neoparamoeba* spp. for 4 weeks was 47% higher than that of fish that were AGD-naïve. While the approach adopted here to assess resistance of Atlantic salmon that were previously affected by AGD was based on a commonly used model for testing efficacy of vaccines (Midtlyng, 2005), a more pronounced level of resistance may have been seen had the challenge period been extended. The substantial reduction in mortality of fish previously exposed to *Neoparamoeba* spp. presented here is in contrast with the findings of Gross et al., (2004b). Results obtained in the latter study may have been influenced by mortalities that occurred during primary infection, possibly introducing bias into the challenge. In addition, the freshwater bath treatment administered after the primary AGD infection was not effective and as a result, fish maintained in sea water after primary exposure remained infected and further mortalities occurred. Consequently, at the time of AGD challenge, fish maintained in seawater were actively infected, introducing bias to the challenge model.

Fish that were previously exposed to *Neoparamoeba* spp. and demonstrated resistance in terms of substantially lower mortality presented similar gill pathology to AGD-naïve fish after challenge. This is in contrast with earlier studies that have reported resolution of gross gill lesions during secondary exposure (Findlay, et al., 1995; Findlay & Munday, 1998). However, in both aforementioned studies, fish were allowed to recover for a period of four weeks in fresh water after primary infection while in the current study, fish were bathed

for 24 hours. A prolonged period of infection may be important for development of resistance in terms of lesion resolution. This was more recently observed in Atlantic salmon that had been constantly exposed to *Neoparamoeba* spp. for 10 weeks (Bridle, et al., 2005). Similarly, the Atlantic salmon from which the positive control serum was obtained displayed a low level of gross pathology and had been exposed to AGD for over 3 months. These observations suggest that resistance can develop over time and had the challenge period been extended in the current study, further resistance in the form of reduced gill pathology may have been seen.

Following AGD challenge, anti-*Neoparamoeba* spp. antibodies were only detected in serum from 50% of the surviving Atlantic salmon that had been previously exposed to *Neoparamoeba* spp.. This suggests that secondary exposure, or the collective duration of primary and secondary exposure may be important for developing a systemic response. Increasing antibody titre is often seen after 4 weeks in fish vaccinated with parasitic antigens. For example, levels of specific antibody in serum after injection with *Cryptobia salmositica* increase substantially in Atlantic salmon after 5 weeks (Mehta & Woo, 2002) and after 6 weeks in rainbow trout (Chin, Glebe & Woo, 2004) post-vaccination. Further, specific antibody titre increases dramatically in channel catfish, 7 weeks post-vaccination with *I. multifiliis* theronts (Wang & Dickerson, 2002). While specific antibody was detectable in only 50% of the resistant population in the current study, individual variation in the development of an adaptive response in fish is common (Lobb, 1987; Akhlaghi, et al., 1996; Steine, Melingen & Wergeland, 2001; Maki & Dickerson, 2003). A modest antibody response has been previously detected in serum of Atlantic salmon affected by AGD (Akhlaghi, et

al., 1996; Gross, et al., 2004a), however in contrast with these studies, antibodies identified here are specific to wild-type amoebae and are possibly directed against epitopes that are associated with virulence of *Neoparamoeba* spp. The binding profile of positive serum was consistent, producing a smear across a broad molecular range, similar to that seen with antibody binding to proteoglycan antigens (Fischer, Haubeck, Eich, Klobe-Busch, Stocker, Stuhlsatz & Greiling, 1996; Yeh, Chen, Li, Espana, Ouyang, Kawakita, Kao, Tseng & Liu, 2005). However, without further analysis, no assumption can be made of the nature of the epitope(s). While clearly detectable by Western blot and flow cytometric analysis, specific antibody was undetectable in the same samples by ELISA suggesting that titre was extremely low. Atlantic salmon serum used for the positive control in both Western blot and ELISA was obtained from a fish that had survived AGD infection for over 3 months and had a significant ELISA optical density at a dilution of 1:100. Binding of the positive control serum is also specific to wild-type *Neoparamoeba* spp. antigen(s) and produced a similar binding profile suggesting that titre may positively correlate with increased duration of infection.

Western blot analysis of cutaneous mucus samples that were taken from fish that had developed a systemic response did not identify anti-*Neoparamoeba* spp. antibodies. Stability of IgM in mucus samples was confirmed by Western blot indicating that the negative result was not influenced by sample degradation, the ability of the rabbit anti-salmon antibody to bind mucosal IgM or the sensitivity of the assay. Specific antibodies against *I. multifiliis* have been detected in cutaneous mucus of channel catfish 3 weeks after bath treatment (Maki & Dickerson, 2003). Similar responses have been documented after i.p.

immunisation of rainbow trout with FITC conjugated keyhole limpet haemocyanin (Cain, Jones & Raison, 2000) or *Flavobacterium psychrophilum* (LaFrentz, LaPatra, Jones, Congleton, Sun & Cain, 2002) with mucosal antibody in all cases measurable in 3 to 4 weeks after immunisation. The antibody response in cutaneous mucus is often substantially lower than the systemic response to a range of antigens (Cain, et al., 2000; LaFrentz, et al., 2002; Maki & Dickerson, 2003). In the current study, failure to detect anti-*Neoparamoeba* spp. antibodies in cutaneous mucus may be the result of very low titre. The cotton swab method used to collect mucus was chosen over a scraping method to reduce the potential level of contamination of mucus with host cells. Further, while the mucus collection method used in this study was successful in that total mucosal IgM could be detected, collecting in a volume of 500 µL may have diluted specific antibody levels to below the limit of detection. The antibody response to *Neoparamoeba* spp. may be more localised in the gill epithelium or gill mucus as gill-associated antibodies can exceed levels of systemic antibody (Lumsden, Ostland, MacPhee & Ferguson, 1995). However, a failure to develop a mucosal antibody response to *Neoparamoeba* spp. must also be considered.

In summary, the results presented here provide further evidence that Atlantic salmon can develop resistance, in terms of reduced mortality, to AGD. Duration of exposure may be significant to the development of resistance and to the detection of a systemic antibody response. Further study of the role of systemic antibody in protection against AGD and characterisation of the target epitope(s) is required and is the focus of ongoing studies.

**Acknowledgements**

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# Chapter 3

# Detection of serum anti-*Neoparamoeba* spp. antibodies in amoebic gill disease-affected Atlantic salmon

***This Chapter has been published, in part, in the Journal of Fish Biology:***

Vincent, B. N., Nowak, B. F. and Morrison, R. N., (2008) Detection of serum anti-*Neoparamoeba* spp. antibodies in amoebic gill disease (AGD)-affected Atlantic salmon (*Salmo salar* L.). *Journal of Fish Biology,* 73, 429-435.

**Abstract**

In some cases, amoebic gill disease (AGD)-affected Atlantic salmon develop a serum antibody response to wild-type *Neoparamoeba* spp.. Here, sera from AGD-affected and AGD-naïve Atlantic salmon were screened against wild-type and cultured *Neoparamoeba* spp. antigens. Only 5 of 103 AGD-affected Atlantic salmon produced detectable antibodies that bound wild-type *Neoparamoeba* spp.. Western blotting revealed two distinctly different binding profiles. Anti-*Neoparamoeba* spp. antibodies present in the serum of four fish bound to periodate oxidation-sensitive antigens across a broad molecular weight range producing a smear. In contrast, binding of antibodies present in the serum of the remaining fish bound two antigenic determinants *Mr* >200 kDa that were periodate oxidation resistant. These data suggest that Atlantic salmon have the capacity to develop a serum antibody response to wild-type *Neoparamoeba* spp. infection although the development of an antibody response with measurable activity in an ELISA is rare.

## Introduction

Amoebic gill disease (AGD) affects sea-farmed salmonids in Tasmania, Australia (Munday, 1986; Munday, et al., 1990; Munday, Lange, Foster, Lester & Handlinger, 1993), Ireland (Rodger & McArdle, 1996; Palmer, et al., 1997) the USA (Kent, et al., 1988), Chile, New Zealand (Munday, et al., 2001) and Scotland (Young, et al., 2007b). In Tasmania, AGD is a particularly serious health concern as recurrent epizootics occur (Munday, et al., 1990). At present, fresh water bathing is the only commercially viable treatment for AGD and contributes 10-20% of production costs (Munday, et al., 2001). Therefore the development of an alternative to freshwater bathing, such as an AGD vaccine, is a priority for many salmon growers.

Several efficacious fish vaccines, including those for vibriosis, yersiniosis and furunculosis (Hastein, Gudding & Evensen, 2005), are composed of whole cell preparations. These vaccines were developed by simply immunising fish with inactivated cells with or without adjuvant. As an alternative to this immunisation approach, host immune sera may be used to identify candidate vaccine antigens. Fish immune sera have been used to identify protective antigens of the protozoan fish parasite *Ichthyophthirius multifiliis*. Immobilization of *I. multifiliis* theronts was first observed *in vitro* using immune sera from fish that had survived an initial *I. multifiliis* infection (Hines & Spira, 1974). Subsequently, it was shown that parasite immobilization is associated with antibody binding to cell-surface ciliary antigens (Clark, et al., 1988; Clark & Dickerson, 1997) and that protection of channel catfish, *Ictalurus punctatus* Rafinesque, against white spot *in vivo* is antibody-mediated (Wang & Dickerson, 2002).

There is preliminary evidence for the development of a serum antibody response in AGD-affected Atlantic salmon (Vincent, Morrison & Nowak, 2006). Therefore this may be exploited to identify wild-type *Neoparamoeba* spp. antigens expressed *in vivo*. In the current study, we opportunistically screened sera from AGD-affected Atlantic salmon with the aim of identifying antigen(s) specific to wild-type *Neoparamoeba* spp.. Sera from very few Atlantic salmon affected by AGD contained anti-*Neoparamoeba* spp. antibodies. Of those sera with demonstrable antibody activity in an ELISA, anti-*Neoparamoeba* spp. antibodies were predominately directed towards cell-surface carbohydrate residues unique to wild-type *Neoparamoeba* spp.. However no causal relationship between serum antibodies and resistance to AGD could be established.

## Materials and methods

*Fish history and blood sampling*

Sera assessed for anti-*Neoparamoeba* spp. antibodies in the current study were taken from a total of 103 AGD-affected and 44 AGD-naïve Atlantic salmon. At present, a source of wild-type *Neoparamoeba* spp. are maintained via co-habitation of AGD-naïve Atlantic salmon with AGD-affected Atlantic salmon (UTAS co-habitation tank) at the University of Tasmania, Australia. The AGD-affected Atlantic salmon assessed for serum anti-*Neoparamoeba* spp. antibodies in the current study were exposed to wild-type *Neoparamoeba* spp., either in the UTAS co-habitation tank or by inoculation of the fish holding systems with wild-type *Neoparamoeba* spp. (Table 3.1). AGD-affected fish included 17 fish

collected from the UTAS co-habitation tank and of these 15 were collected as they became moribund from AGD. The remaining two fish from the UTAS co-habitation tank were larger than the tank cohort (range 80-150 g) and at the time of sampling weighed 580 and 340 g. These fish are herein referred to as fish one and fish two respectively. Due to their size, fish one and fish two were easily observed in the tank and it was estimated that fish one and fish two had been in the UTAS co-habitation tank for six and four months respectively. Fish transferred to the UTAS co-habitation tank generally become moribund from AGD within four weeks. Blood was taken from fish one once while fish two was bled four times at 4 week intervals during the 4 month period. A further two groups of AGD-affected Atlantic salmon were assessed for serum anti-*Neoparamoeba* spp. antibodies. These included 23 Atlantic salmon exposed to wild-type *Neoparamoeba* spp (500 cells/L) for 34 days and 63 AGD-affected Atlantic salmon exposed to wild-type *Neoparamoeba* spp. (1152 cells/L) for 72 days in the experiment described by Bridle Carter, Morrison, and Nowak, (2005). *Neoparamoeba* spp. are obligate marine organisms, therefore sera from 44 Atlantic salmon maintained only in fresh water were tested to assess if natural antibodies present in the sera of AGD-naïve Atlantic salmon bound amoeba antigens. The holding conditions and duration of exposure of Atlantic salmon to wild-type *Neoparamoeba* spp. of the fish from which the sera were assessed in the current study are summarised in Table 3.1.

**Table 3.1.** Serum from AGD-affected and AGD-naïve Atlantic salmon was assessed for anti-*Neoparamoeba* spp., antibodies. The number of fish sampled, the duration of exposure to wild-type *Neoparamoeba* spp. and fish holding conditions are summarised.

| Number of fish | Mode of exposure | Weeks exposed | Salinity (‰) | Water Temperature (°C) |
|---|---|---|---|---|
| 17 | Co-habitation | 3-24[+] | 35 | 16 |
| 23 | Inoculation (500 cells/L) | 4.8 | 35 | 16 |
| 63 | Inoculation[1] (1152 cells/L) | 10.3 | 35 | 16 |
| 44 | Not exposed | N/A | 0 | 14-16 |

[1] Fish sampled from the experiment described by (Bridle, et al., 2005), [+] Estimated exposure time, N/A – not applicable.

*Cultured and wild-type Neoparamoeba spp.*

Amoebic gill disease (AGD) of Atlantic salmon is predominantly associated with the amphizoic marine amoebae, *Neoparamoeba perurans* (Young, et al., 2007a; Young, et al., 2007b). While *N. pemaquidensis* and *N. branchiphila* have also been isolated from AGD-affected gill tissues of Atlantic salmon by culture (Dyková, et al., 2005a) neither *N. pemaquidensis* nor *N. branchiphila* have been identified in association with AGD lesions. Despite this, amoebae isolated from gill tissues of AGD-affected Atlantic salmon may include all the above-mentioned *Neoparamoeba* species and gill-derived amoebae are therefore described as wild-type *Neoparamoeba* spp.. Wild-type *Neoparamoeba* spp. were isolated as described by Morrison, Crosbie and Nowak (2004) from AGD-affected Atlantic salmon maintained in the UTAS co-habitation tank. Clonal strains of cultured *N. pemaquidensis* and *N. branchiphila* tested to date are

avirulent (Kent, et al., 1988; Howard, Carson and Lewis, 1993; Findlay, 2001; Morrison, Crosbie, Cook, Adams and Nowak, 2005; Vincent, Adams, Crosbie, Nowak and Morrison, 2007). Therefore, to discriminate between reactive epitope(s) of cultured and wild-type *Neoparamoeba* spp., two previously characterised clonal strains (Dyková et al., 2005) of cultured *Neoparamoeba* sp. were used as negative controls. These were *N. pemaquidensis* (NP251002) (Morrison, et al., 2005) isolated from AGD-affected Atlantic salmon, and *N. branchiphila* (SEDMH1) isolated from the sediment of Macquarie Harbour, Tasmania. Amoebae were maintained on seawater malt yeast agar consisting of 75% (v/v) coarse-filtered seawater (35‰), 25% (v/v) distilled water, 0.01% (w/v) Malt, 0.01% (w/v) yeast (Oxoid, Hampshire, England) and 2% (w/v) Bacto agar (Becton, Dickson and Co., USA). Cells were harvested by washing the agar with sterile sea water. Cells were concentrated by centrifugation at 500× *g* for 5 min and enumerated by hemacytometer. Amoebae were washed twice with phosphate buffered saline (PBS, pH 7.2) and the cell pellet was stored at -80°C.

**Presence of serum anti-*Neoparamoeba* spp. antibodies**

*SDS-PAGE and Western blot*

The binding of Atlantic salmon serum antibodies to cultured or wild-type *Neoparamoeba* spp. antigens was assessed by Western blotting. Wild-type *Neoparamoeba* spp. were thawed, resuspended in buffer containing β-mercaptoethanol and reduced by boiling for 10 min. Reduced antigen was centrifuged at 16 000 × *g* and the supernatant stored at - 20°C. Amoebae antigens were separated by SDS-PAGE through 6% polyacrylamide gels. In addition, cutaneous mucus was obtained from AGD-naïve Atlantic salmon by gently

scraping the skin with the edge of a glass slide. Mucus was centrifuged for 5 min at 500 × *g* to separate mucus from cellular debris, reduced, as outlined above, centrifuged and the supernatant stored at -20°C. Mucus was included as an antigen control at 14 µg total protein/lane. Antigens were transferred to nitrocellulose membrane (Hybond-C extra, Amersham Biosciences, UK) using a semi-dry transfer apparatus (Hoefer Scientific Instruments, San Francisco, California, USA) and blocked in 1× casein solution (Vector, Burlingame, California, USA). Blocking and antibody incubation steps were for 30 min and in between incubation steps, membranes were washed 3 × 4 min with tris-buffered saline (TBS, pH 7.2). Atlantic salmon test serum, serum previously identified to contain anti-*Neoparamoeba* spp. antibodies (Vincent, et al., 2006), or normal Atlantic salmon serum was applied at 1:500 in 1× casein solution and bound antibodies were detected with rabbit anti-salmon IgM at 1:500 followed by alkaline phosphatase (AP)-conjugated sheep anti-rabbit IgG (Chemicon, Boronia, Australia) at 1:2000. Following the final antibody incubation, membranes were washed 3× in TBS and then 1× in 100 mM tris (pH 9.5) for 5 min. All incubation and wash steps were conducted at 20°C. Western blots were developed by enhanced chemiluminescence (ECL) using DuoLuX (Vector), Kodak BioMax Light Film and Kodak GBX developing and fixing reagents (Sigma, Castle Hills, NSW, Australia) following the manufacturer's instructions. Binding of the polyclonal rabbit anti-salmon IgM to purified and crude Atlantic salmon serum IgM was initially confirmed by Western blotting (refer to Chapter 2, Figure 2.8).

*Sodium periodate oxidation of carbohydrate epitope(s) of wild-type Neoparamoeba spp.*

Wild-type *Neoparamoeba* spp. antigens were transferred to nitrocellulose membrane as outlined above. From the same membrane, bound antigens on adjacent strips were either oxidised with 20 mM sodium periodate (Merck Pty Ltd., Victoria) and 50 mM sodium borohydride (Sigma-Aldrich) or incubated in 50 mM sodium acetate (pH 4.5, Sigma) following the method outlined by Woodward et al., (1985). Membranes were then washed 3× with PBS, probed and developed as above.

*Enzyme-linked immunosorbent assay (ELISA)*

Binding of anti-*Neoparamoeba* spp. antibodies was quantified by an ELISA. Wild-type *Neoparamoeba* spp. were isolated as outlined above, stored at -20°C and re-suspended in PBS. Wild-type *Neoparamoeba* spp. were sonicated on ice until complete disruption of cells was observed. The sonicated suspension of wild-type *Neoparamoeba* spp. antigens was then centrifuged for 10 min at 16 000× g and the supernatant removed. Protein concentration of the sonicated *Neoparamoeba* spp. supernatant was determined by colorimetric assay (Pierce, Rockford, USA). The ELISA antigen was then divided into aliquots suitable for coating a single 96-well plate and stored at -20° C. Optimal conditions for the ELISA were determined empirically. Briefly, antigen was thawed, re-suspended in coating buffer (50 mM $NaHCO_3$, pH 9.5) and 96-well flat-bottom plates (Sarstedt, Australia) were coated with 50 µL sonicated wild-type *Neoparamoeba* spp. (0.24 µg total protein/well) in coating buffer at 4°C overnight. Excess antigen was discarded and wells were blocked for 30 min at 37°C with 0.3 %

casein-PBS (Sigma). Serum samples were serially diluted in 0.3 % casein-PBS in duplicate from 1:100 to 1:3200, (50 µL/well) and plates were incubated for 1 h at 20°C. Bound antigen was detected with polyclonal rabbit anti-salmon IgM at 1:500 and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma) at 1:1000 for 30 min at 37°C. The reaction was developed with 50 µL o-phenylenediamine (OPD) (Sigma) and stopped with an equal volume of 3M HCl. AGD-naïve Atlantic salmon serum was included in each assay as the negative control. AGD-naïve serum was pooled from five fish that were maintained in fresh water and therefore AGD-naïve as *Neoparamoeba* spp. are obligate marine organisms. Positive and negative control serum was titrated from 1:100 to 1:3200 on each plate in duplicate. Following the method described by Arkoosh and Kaattari (1990), further analysis was performed on the data obtained from serum samples containing anti-*Neoparamoeba* spp. antibodies with activity measurable by the ELISA. Arkoosh and Kaattari (1990) describe a method to calculate antibody activity by utilising a standard positive control serum across all ELISAs and correcting for variation in optical density that is inherent between assays. This method enables comparison of antibody activity across assays.

*Immunocytochemistry and flow cytometry*

Wild-type amoebae were fixed in seawater Davidson's fixative (SWD) for 1 h at 20°C and washed by four cycles of re-suspending cells in PBS and concentrating cells by centrifugation at 500 × *g* for 5 min. Amoebae were placed in the wells of 96-well U-bottomed microplates (Sarstedt) and blocked in 0.1% BSA-PBS for 30 min at 4°C. Cells were probed with AGD-naïve Atlantic salmon serum or serum that contained anti-*Neoparamoeba* spp. measured by both the ELISA and

Western blotting. Bound antibodies were detected with rabbit anti-salmon IgM at 1:100 and FITC-conjugated sheep anti-rabbit IgG (Chemicon) at 1:50. Cells were washed 3× in PBS following each antibody incubation step. A sub-sample of cells was photographed (Leica DC300F, Leica Microsystems, Wetzlar, Germany) using light and epi-fluorescent microscopy. Using the remaining probed cells, the proportion of wild-type *Neoparamoeba* spp. expressing epitope(s) to which the salmon anti-*Neoparamoeba* spp. antibodies bound was quantified by flow cytometry (Coulter Epics, Beckman Coulter, USA). Ten thousand cells were assessed per treatment and data were analysed using WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, California, USA).

*Glycoprotein staining of wild-type Neoparamoeba spp. antigens*

ProteoProfile PTM markers (Sigma, St Louis, MO, USA) and two lanes of wild-type *Neoparamoeba* spp. antigens (8 x $10^4$ cell equivalents/lane) were separated through a 4-15% polyacrylamide gel. One lane of wild-type *Neoparamoeba* spp. antigen was cut from the gel, silver stained and photographed. The remaining gel was stained for glycoproteins using the GlycoProfile III fluorescent glycoprotein detection kit following the manufacturer's instructions (Sigma). Following staining, the glycoprotein stained gel was viewed under UV light and photographed.

## Results

*Presence of serum anti-*Neoparamoeba *spp. antibodies*

By Western blotting, antibodies that bound wild-type *Neoparamoeba* spp. antigens were detected in the sera of five out of 103 fish. These included samples obtained from two fish from the UTAS co-habitation tank (fish one and fish two) and three fish from the experiment described by Bridle et al., (2005). Fish one and fish two serum antibodies were specific to wild-type *Neoparamoeba* spp. yet distinctively different binding profiles were produced in Western blot. Binding of serum antibodies of fish one by Western blot produced two bands >200 kDa and serum antibodies of fish two produced a smear across a broad molecular weight range (Fig. 3.1). Sodium periodate oxidation of wild-type antigens was performed to assess the binding of anti-*Neoparamoeba* spp. antibodies to peptide or carbohydrate epitope(s). Antibodies present in the serum of fish one were directed towards epitope(s) that were not sensitive to periodate oxidation while antibodies in the serum of fish two failed to bind periodate-treated wild-type *Neoparamoeba* spp. antigens (Fig. 3.1). Similarly, antibodies present in the three sero-positive fish from the experiment described by Bridle et al., (2005) were specific to wild-type *Neoparamoeba* spp. and produced a smear across a broad molecular weight range. In addition, these antibodies failed to bind wild-type *Neoparamoeba* spp. antigens after periodate oxidation (data not shown). In the experiment described by Bridle et al., (2005), β-glucan diets were administered and there was no effect of diet on the susceptibility of Atlantic salmon to AGD. The sero-positive fish identified here were from the control group and were fed commercial Atlantic salmon feed. The five sero-positive samples were also screened against Atlantic salmon cutaneous mucus supernatant obtained from

AGD-naïve Atlantic salmon to identify potential cross-reactivity with normal flora residing in the host mucus and no binding was observed (data not shown). In addition to the normal serum controls ran in parallel with the test sera, sera from a further 44 AGD-naïve Atlantic salmon were assessed for presence of natural antibodies that may bind *Neoparamoeba* spp. antigens. Antibodies present in the sera of 44 AGD-naïve Atlantic salmon did not bind wild-type or cultured *Neoparamoeba* spp. antigens (data not shown).

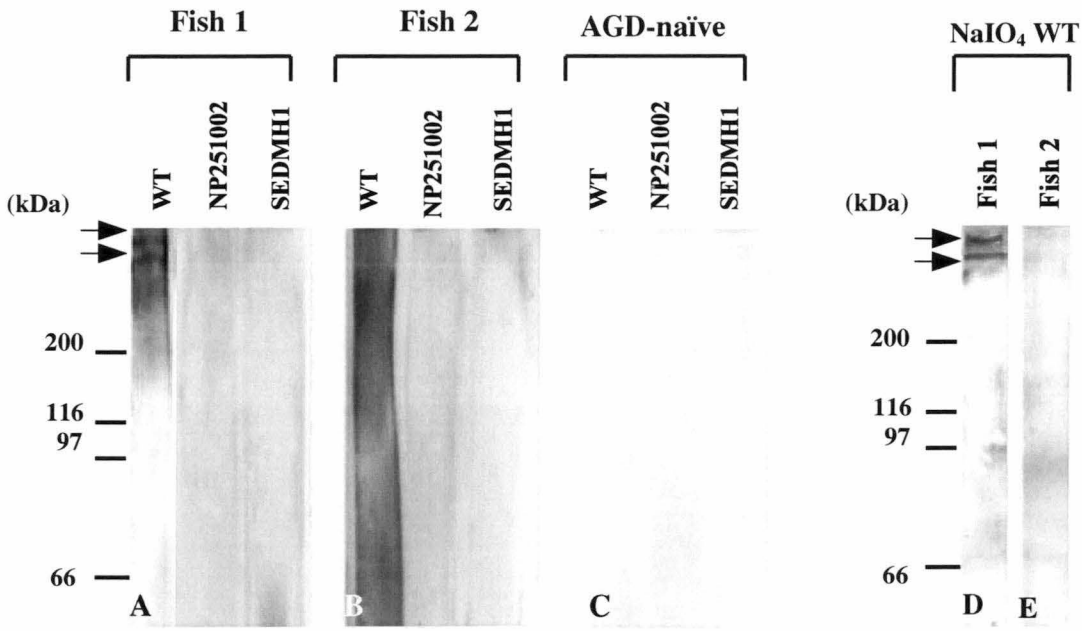


**Figure 3.1**. Binding of serum anti-*Neoparamoeba* spp. antibodies from fish one and fish two to wild-type *Neoparamoeba* spp. (WT) produces distinctly different profiles. Anti-*Neoparamoeba* spp. antibodies in fish one serum bind two bands (arrows) >200 kDa (A) of wild-type antigen (WT) and these epitope(s) are not sensitive to periodate oxidation (D). In contrast, binding of anti-*Neoparamoeba* spp. antibodies in fish two serum produce a smear across a broad molecular range (B) and these epitope(s) are sensitive to periodate oxidation (E). Antibodies present in the serum of fish one and fish two do not bind cultured *N. pemaquidensis* (NP251002) or *N. branchiphila* (SEDMH1) and antibodies present in serum from AGD-naïve fish do not bind wild-type or cultured *Neoparamoeba* spp. (C). Antigens were reduced in sample buffer containing β-mercaptoethanol, separated through a 6% polyacrylamide gel and each lane was loaded with 8 x $10^4$ cell equivalents/lane. Wild-type antigens were transferred to nitrocellulose and treated with sodium periodate ($NaIO_4$WT) (lanes D and E). Membranes were probed with serum from fish one (A and D), fish two (B and E) and normal salmon serum (C). Bound antibodies were detected with rabbit anti-salmon IgM, AP-conjugated sheep anti-rabbit IgG and chemiluminescence. AGD-naïve serum was pooled from five fish held in fresh water and therefore was from AGD-naïve fish.

The only samples containing anti-*Neoparamoeba* spp. antibodies with measurable activity according to the method outlined by Arkoosh and Kaattari (1990) by an ELISA were those of fish one and fish two. Due to the larger volume of serum attained from fish two, fish two serum was used as the positive control. Binding of anti-*Neoparamoeba* spp. antibodies present in the serum of fish one produced an optical density similar to the positive control sera (Fig. 3.2). At a serum dilution of 1:100, the mean optical density produced by the AGD-naïve serum was 0.19 (± SEM 0.00), at the same serum dilution, antibodies present in the serum of fish one and fish two (the positive control serum) produced optical densities of 0.79 (± SEM 0.03) 0.68 (± SEM 0.02) respectively. Whilst the optical density produced at the serum dilution of 1:100 was higher for fish one, anti-*Neoparamoeba* spp., the antibody activity of both fish one and fish two serum was equal at 7.7 units/µl of serum. Further analysis of anti-*Neoparamoeba* spp. antibodies was restricted to the serum samples from fish one and fish two with measurable antibody activity.

*Immunocytochemistry and flow cytometry*

Fish one and fish two anti-*Neoparamoeba* spp. antibodies bound cell-surface epitope(s) of wild-type *Neoparamoeba* spp. producing intense fluorescence around the cell margin (Fig. 3.3). Binding of fish one and fish 2 anti-*Neoparamoeba* spp. antibodies was quantified by flow cytometry and fluorescence intensity significantly greater than the normal serum control was observed. Fluorescence intensity ten-fold greater than the normal serum control was produced by the binding of fish one and fish 2 antibodies to 94.7% and 94.6% of the wild-type *Neoparamoeba* spp. analysed respectively (Fig. 3.3).

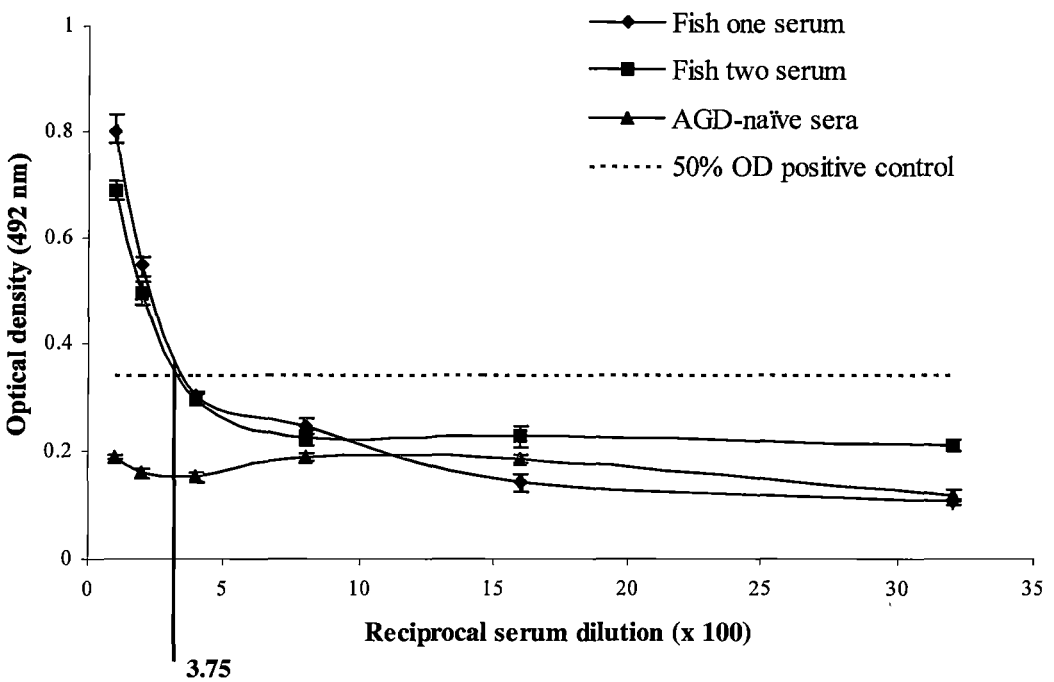


**Figure 3.2.** Anti-*Neoparamoeba* spp. antibodies present in serum of fish one and fish two produce a significant optical density in ELISA. Wells were coated with sonicated wild-type *Neoparamoeba* spp. antigen (0.24 μg total protein/well). Serum was titrated from 1:100 to 1:3200 in triplicate. Bound antibodies were detected with rabbit anti-salmon IgM, HRP-conjugated goat anti-rabbit IgG and OPD. Titration curves represent the mean ± SEM of the optical density at 492nm. AGD-naïve serum was pooled from five Atlantic salmon held in fresh water and therefore was from AGD-naïve fish. The dilution used to calculate the antibody activity of fish one and fish two serum (extrapolated from the optical density (OD) that represented 50% of the OD of the positive serum control) were equal for both fish at 1:375. Antibody activity was calculated by the method described by Arkoosh and Kaattari (1990).

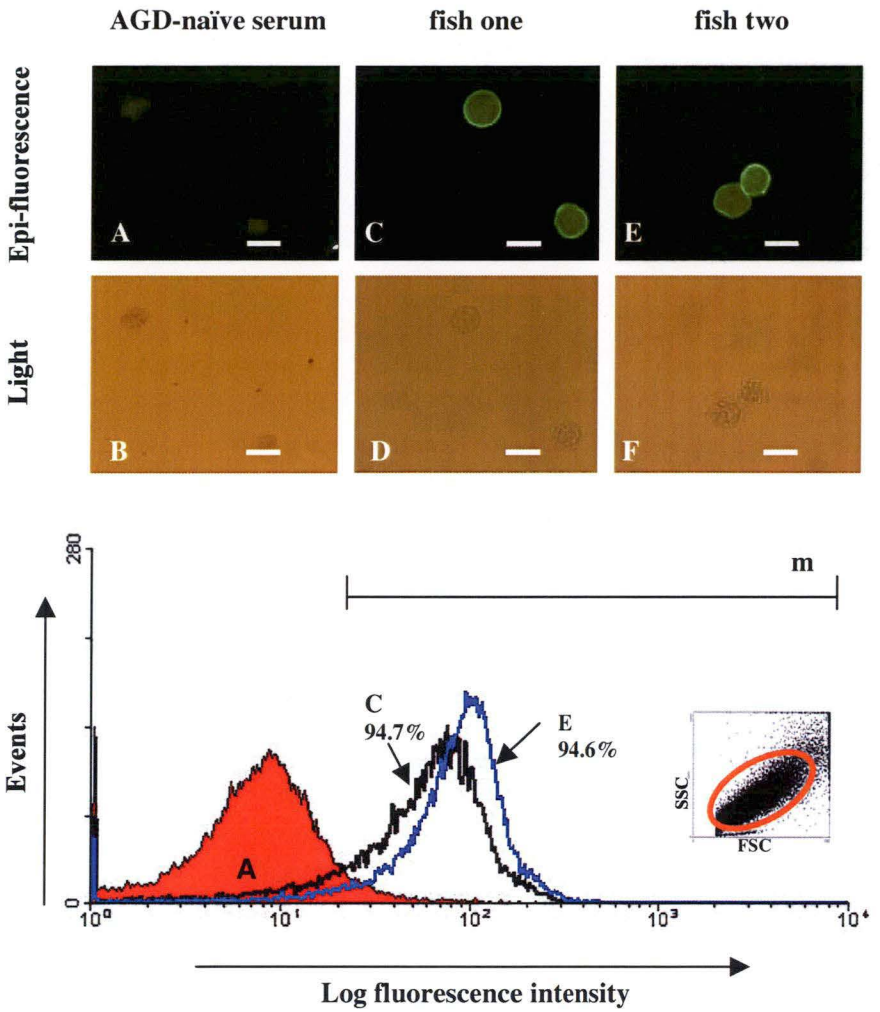


**Figure 3.3.** Anti-*Neoparamoeba* spp. antibodies present in the serum of fish one and fish two bind cell-surface epitope(s) of wild-type *Neoparamoeba* spp.. Adjacent images are the corresponding light micrographs. Fluorescence intensity significantly higher than the AGD-naïve serum control (A) is produced by binding of fish one (C) and fish two (E) anti-*Neoparamoeba* spp. antibodies to 94.7% and 94.6% of the analysed wild-type *Neoparamoeba* spp. cell suspension. Wild-type *Neoparamoeba* spp. were fixed and probed with AGD-naïve serum, fish one or fish two serum. Bound antibodies were detected with rabbit anti-salmon IgM and FITC-conjugated sheep anti-rabbit IgG. The shaded area of the histogram represents cells probed with AGD-naïve serum (A). Data presented includes cells within the gated region shown in the dot-plot (inset). Flow cytometry data were analysed and presented using WinMDI 2.8 software. Scale bars = 50 μm.

*Glycoprotein staining of wild-type* Neoparamoeba *spp antigens*

Wild-type *Neoparamoeba* spp. glycoproteins were observed under UV illumination after glycoprotein staining of the gel. Glycoprotein staining of wild-type *Neoparamoeba* spp. antigens produced a smear across a broad molecular weight range. Silver staining of wild-type *Neoparamoeba* spp. antigens similarly presented as a smear with no distinguishable banding (Fig. 3.4).

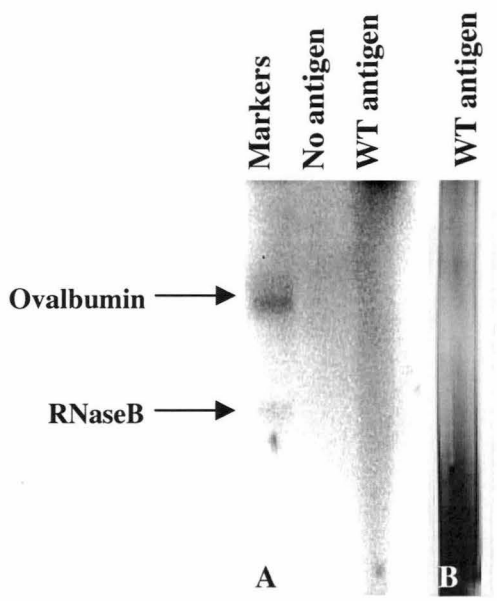


**Figure 3.4.** Wild-type *Neoparamoeba* spp. glycoproteins present as a smear across a broad molecular weight range. Amoebae antigens ($8 \times 10^4$ cell equivalents) and glycoprotein markers were separated by SDS-PAGE through a 4-15% gel. One lane of wild-type *Neoparamoeba* spp. antigen was separated and silver stained (B). The remaining gel was stained for glycoproteins (A). The glycoproteins ovalbumin (45 kDa) and RNaseB (17 kDa) are indicated. The glycoprotein stained gel section (A) was viewed and photographed under UV light.

**Discussion**

Sera from AGD-affected and AGD-naïve Atlantic salmon were screened for the presence of anti-*Neoparamoeba* spp. antibodies against wild-type and cultured *Neoparamoeba* spp. antigens, however few sero-positive samples were identified. The predominant antibody-binding profile observed here presented as a smear by Western blotting. Similarly, anti-*Neoparamoeba* spp. antibodies detected in the sera of AGD-affected Atlantic salmon in the study described by Vincent et al., (2006) produced a smear in Western blot. Presentation of a smear by Western blotting is characteristic of antibody binding to carbohydrate residues. For example, antibody binding to the proteoglycan agrin (Groffen, Ruegg, Dijkman, van de Velden, Buskens, van den Born, Assman, Monnens, Veerkamp and van den Heuvel, 1998), carbohydrate antigens of the mould, *Aspergillus versicolor*, (Rydjord, Hetland and Wilker, 2005) and mucin-like glycoproteins (Hong, Jang, Kong, Song, Park, Kim, Chung, Lee, Paik and Chung, 2001) produced a smear by Western blotting. Certainly, the profile produced by glycoprotein staining suggests that wild-type *Neoparamoeba* spp. express abundant glycosylated molecules. The loss of antibody binding following periodate oxidation, as seen here, is indicative of antibody binding to carbohydrate residues (Woodward, Young and Bloodgood, 1985).

Monoclonal antibodies produced against wild-type *Neoparamoeba* sp. are predominately directed towards cell-surface carbohydrate epitopes (Villavedra, Lemke, To, Broady, Wallach and Raison, 2007). Similarly, results presented here suggest that cell-surface carbohydrate epitope(s) of wild-type *Neoparamoeba* spp. may also be immunodominant in Atlantic salmon. In contrast to the

predominant antibody binding profile that presented as a smear by Western blotting, the serum antibodies of fish one bound two high molecular weight epitope(s) of wild-type *Neoparamoeba* spp.. These high molecular weight epitope(s) are likely to be peptide(s) as antibody binding was observed after sodium periodate oxidation. Ideal vaccine candidate antigen(s) for an AGD vaccine would be peptide(s) that are easily produced by recombinant DNA technology. Further characterisation of these high molecular weight peptide epitope(s) is warranted and these epitope(s) are currently under further investigation in our laboratory.

In some cases, natural antibodies may bind antigens of pathogens. For example natural antibodies present in serum of goldfish, *Carassius auratus* L., bind the A-layer proteins of *Aeromonas salmonicida* (Sinyakov, Dror, Zhevelev, Margel and Avtalion, 2002) and natural antibodies in the serum of rainbow trout bind the monogenean *Discocotyle sagittata* (Rubio-Godoy, Sigh, Buchmann and Tinsley, 2003). Serum antibodies from 44 AGD-naïve Atlantic salmon did not bind wild-type or cultured *Neoparamoeba* spp. antigens suggesting that natural antibodies do not bind *Neoparamoeba* spp. antigen(s). However, AGD-affected fish assessed in the current study were not sampled prior to exposure to *Neoparamoeba* spp.. Therefore it cannot be discounted that the serum antibodies described here may be natural antibodies.

The development of a detectable serum antibody response in Atlantic salmon to wild-type *Neoparamoeba* spp. may rely on a range of factors. Here, fish one and fish two developed a serum antibody response with measurable activity after

exposure to wild-type *Neoparamoeba* spp. by co-habitation for an estimated six and four months respectively. In the study by Vincent et al., (2006), 50% of AGD-affected Atlantic salmon developed a serum antibody response 5 weeks following secondary exposure to wild-type *Neoparamoeba* spp. (9 weeks after initial exposure). While this suggests that the duration of exposure may be important for the development of a serum antibody response to *Neoparamoeba* spp., just 3 of the 63 fish exposed to wild-type *Neoparamoeba* spp. for 72 days in the experiment described by Bridle et al., (2005) developed a detectable serum antibody response. Entrapment of *Neoparamoeba* spp. within interlamellar vesicles (Adams and Nowak, 2001) may provide the environment for interaction with immune-like cells. MHC $II^+$ cells are present throughout AGD lesions of Atlantic salmon gill tissues (Morrison, Koppang, Hordvik and Nowak, 2006). Antigen processing may be restricted to opportunistic interactions between wild-type *Neoparamoeba* spp. entrapped in interlamellar vesicles and MHC $II^+$ cells and this may influence the development of a serum antibody response. While fish one and fish two survived for a significant length of time in the UTAS co-habitation tank, these are isolated observations and results presented here provide no evidence to suggest that serum antibodies may be associated with AGD resistance in Atlantic salmon.

It is interesting that antibodies present in the sera of five fish were detectable by Western blotting yet in an ELISA, antibody activity was only measurable in the serum of fish one and fish two. The ELISA conditions applied here and by Vincent et al., (2006) were the same and in both instances, antibodies were detectable by Western blotting but not by ELISA.. In all cases, negative and

positive control sera were included on each plate and titration curves were observed for the positive control serum in each ELISA. Processing of wild-type antigen may damage or eliminate reactive epitope(s). However, each ELISA assay was conducted with an independent aliquot of wild-type *Neoparamoeba* spp. antigen from the same antigen pool. Furthermore, fish two antibodies were measurable by the ELISA and appear to bind the same carbohydrate epitope(s) as the three sero-positive fish from the experiment described by Bridle et al., (2005). Given this, the failure to detect antibodies by ELISA suggests that denaturation prior to Western blotting may enhance access to the relevant epitopes or that antibody levels are simply very low.

In summary, 2 of 103 AGD-affected Atlantic salmon developed a serum antibody response with measurable activity towards cell-surface epitope(s) of wild-type *Neoparamoeba* spp.. Carbohydrate epitope(s) of wild-type *Neoparamoeba* spp. appear to be immunodominant in Atlantic salmon and the development of anti-peptide antibodies specific to wild-type *Neoparamoeba* spp. is, at this point, an isolated finding. The current study has focused on Atlantic salmon that were experimentally affected by AGD under relatively aggressive infection conditions in comparison to that experienced by sea-cage cultured Atlantic salmon. Atlantic salmon cultured in Southern Tasmania are exposed to wild-type *Neoparamoeba* spp. multiple times throughout the grow-out cycle and AGD is closely regulated by fresh water bathing. The duration of exposure (or multiple exposures) to wild-type *Neoparamoeba* spp. appears to be important for the development of a serum antibody response in AGD-affected Atlantic

salmon and screening sera from sea-farmed Atlantic salmon for anti-*Neoparamoeba* spp. antibodies may identify a more diverse range of antigens.

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# Chapter 4

# Cell-surface carbohydrate antigen(s) of wild-type *Neoparamoeba* spp. are immunodominant in sea-cage cultured Atlantic salmon (*Salmo salar* L.) affected by amoebic gill disease (AGD)

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**Abstract**

A small proportion of Atlantic salmon experimentally affected by amoebic gill disease (AGD) develop a serum antibody response to wild-type *Neoparamoeba* spp.. These antibodies bind cell-surface epitope(s) and in most cases the epitope(s) are sensitive to sodium periodate oxidation. Here, blood was obtained from Atlantic salmon after 8, 10 and 13 months of sea-cage culture and assessed for the presence of anti-*Neoparamoeba* spp. (anti-NP) antibodies. Generally, an increase in the proportion of fish developing a detectable antibody response to wild-type *Neoparamoeba* spp. was seen over time in culture. Reflected by the number of freshwater bath treatments administered, the triploid and diploid fish may have been affected by AGD multiple times. While the interval between bath treatments increased over time in culture this corresponded to the seasonal reduction in water temperature at the culture site. A further group of putatively AGD-resistant Atlantic salmon broodstock was sampled at 15 months after transfer to sea and anti-NP antibodies were detected in 81% of these samples. The broodstock did not present any gross gill pathology and had not required freshwater bath treatment for over 250 days. Anti-NP antibodies in all sero-positive fish identified here bound cell-surface carbohydrate antigens, however an antibody titre was not detected in any samples by ELISA. Results presented here provide further evidence for the development of an antibody response in AGD-affected Atlantic salmon and that carbohydrate epitopes of wild-type *Neoparamoeba* spp. are immunodominant in Atlantic salmon.

**Introduction**

Protection against fish parasites has, in some instances, been associated with serum antibodies. Antibody-mediated protection has predominantly been associated with the endoparasitic haemoflagellates *Cryptobia salmositica* (see Chin and Woo, 2005) and *Trypanosoma carassii* (see Lischke, Klein, Stierhof, Hempel, Mehlert, Almeida, Ferguson and Overath, 2000). Resistance to the protozoan parasites *Ichthyophthirius multifiliis* and *Cryptocaryon irritans* that cause white spot disease is associated with serum and skin-associated (or mucosal) antibodies (Wang and Dickerson, 2002; Xu and Klesius, 2002; Luo, Xie, Zhu and Li, 2006; Yambot and Song, 2006). Moreover there is evidence to suggest that serum antibodies may be associated with protection against ectoparasites of fish. For example, protection of rainbow trout against the monogenean *Discocotyle sagittata* (see Rubio-Godoy, Sigh, Buchmann and Tinsley, 2003a; Rubio-Godoy, Sigh, Buchmann and Tinsley, 2003b) and protection of the tomato clown-fish, *Amphiprion frenatus* Bloch, against the ectoparasitic dinoflagellate *Amyloodinium ocellatum* has in some cases been associated with serum antibodies (Cobb, Levy and Noga, 1998). Cross-protection of goldfish, *Carassius auratus* L., immunised with *I. multifiliis* or the non-pathogenic ciliate *Tetrahymena pyriformis* against the ectoparasites *Oodinium pillularis*, *Trichodina* sp., *Ichthyobodo necatrix* and *Chilodenella cyprini* is associated with elevated mucus and plasma antibodies (Ling, Sin and Lam, 1993). Further, a reduction in metamorphosis of the parasitic larval stage of the freshwater mussel *Lampsilis reeveiana* is associated with the presence of serum antibodies in largemouth bass, *Micropterus salmoides* Lacepiede, (Dodd, Barnhart, Rogers-Lowery, Fobian and Dimock, 2006).

The obligatory marine amoebae, *Neoparamoeba* spp. cause amoebic gill disease (AGD) of Atlantic salmon (Adams and Nowak, 2004a; Dyková, Nowak, Crosbie, Fiala, Pecková, Adams, Macháčková and Dvořáková, 2005). Atlantic salmon appear to develop resistance to AGD in terms of reduced gill pathology (Findlay, Helders, Munday and Gurney, 1995; Findlay and Munday, 1998; Bridle, Carter, Morrison and Nowak, 2005) and increased survival (Vincent, Morrison and Nowak, 2006). However, evidence to suggest that a humoral immune response may be protective in Atlantic salmon affected by AGD is lacking. Serum antibodies have been detected in some Atlantic salmon demonstrating resistance to AGD in terms of increased survival and/or low-level gill pathology (Vincent, et al., 2006; Vincent, Nowak and Morrison, 2008).

Atlantic salmon cultured in Southern Tasmania, Australia, are affected by AGD and the disease is most prevalent during summer months (Clark and Nowak, 1999; Adams and Nowak, 2003). To alleviate this condition, fish are treated with fresh water and this treatment may be repeated multiple times throughout the grow-out period. Atlantic salmon that are experimentally exposed to *Neoparamoeba* spp. for a prolonged period and/or exposed multiple times develop a serum antibody response (Vincent, et al., 2006; Vincent, et al., 2008) and this suggests that sea-cage cultured Atlantic salmon may develop a serum antibody response to wild-type *Neoparamoeba* spp. over the culture period. Antibodies that bind cultured *Neoparamoeba pemaquidensis* have been detected in the serum of sea-farmed Atlantic salmon (Gross, Carson and Nowak, 2004) however the reactivity of these antibodies with wild-type antigen was not

determined. During the current study, blood from sea-farmed Atlantic salmon was screened for anti-*Neoparamoeba* spp. antibodies to 1) identify if sea-farmed Atlantic salmon developed an antibody response to wild-type *Neoparamoeba* spp. and, if so, 2) to use these sera to identify potential vaccine candidates. Results presented here indicate that cultured Atlantic salmon develop an antibody response to wild-type *Neoparamoeba* spp. and antibodies are directed towards cell-surface carbohydrate epitope(s).

## Materials and methods

*Fish history and sampling*

The gross sign of AGD in Atlantic salmon is raised white patches on gill surfaces. AGD-like lesions assessed by gross observation are in many cases associated with *Neoparamoeba* spp., however assessment may be over-estimated as some AGD-like lesions are not associated with *Neoparamoeba* spp. (Adams, Ellard and Nowak, 2004). Based on this gross sign, the Huon Aquaculture Company Pty. Ltd. (HAC) in Dover, Tasmania, applies a scoring system as a presumptive diagnosis of the level of AGD. Assessment of AGD-like lesions by gross observation is described as clear, with a gill score of 0, to heavy, that is assigned a gill score of 3 (Adams and Nowak, 2003). On-farm monitoring of AGD is regularly performed by assessing the gross gill pathology of a sub-population of fish from each pen. Freshwater bath treatments are administered when the average gill score for the pen approaches a level of light-medium with a gross gill score above 1.5. Freshwater bathing involves the transfer of fish to an adjacent pen containing a liner of fresh water for 3-4 hours.

A total of 175 fish were sampled from HAC. These fish included sea-cage cultured triploid and diploid Atlantic salmon and a group of broodstock that demonstrated resistance to AGD in terms of low, or no, gross gill pathology. The broodstock were the first progeny of a group of Atlantic salmon that were previously selected by HAC as being putatively resistant to AGD in terms of gross gill pathology. Fish were further selected from this population on the basis of gross gill pathology on 2 occasions and fish showing the gross sign of AGD were removed from the population. The broodstock was maintained at the same farm site as commercial culture pens that required freshwater bathing for AGD multiple times (J. Wells, HAC, pers. com.). Induction of triploidy does not guarantee 100% success (Galbreath, Adams, Sherrill and Martin, 2006) and while this group of fish is termed triploid, testing to confirm this was not carried out and a mixed ploidy population may exist. Table 4.1 summarises the number of months of sea-cage culture at the time of sampling, sampling month, pen allocation, number of freshwater baths administered, days elapsed post-last bath, average water temperature for the month of sampling, number of fish that were sero-positive and the total number of fish sampled.

Blood was taken from triploid and diploid Atlantic salmon on 3 occasions, after 8, 10 and 13 months of sea-cage culture. The triploid fish were maintained in two independent pens (pens 1 and 2) and 10 fish from each pen was sampled on each occasion. The diploid fish were initially maintained in a single pen (pen 3) and 20 fish from this pen were sampled after 8 and 10 months of sea-cage culture. Following the second sampling at 10 months, the diploid fish were split across two pens (pens 3a and 3b) and for the final sampling at 13 months, 10 fish from

each pen were sampled. Blood was taken from 55 of the 75 AGD-resistant fish on one occasion after they had been in sea-cage culture for 15 months. Triploid and diploid Atlantic salmon were terminally anaesthetised while the AGD-resistant fish were anaesthetised for blood sampling only and in all cases fish were anaesthetised using clove oil (0.02% w/v). Blood was taken from the caudal vein and stored in heparinised (triploid and diploid) and non-heparinised tubes (AGD-resistant) on ice overnight. The following day, blood was centrifuged at 1000× *g* for 10 min and the plasma (triploid and diploid) and serum (AGD-resistant) was stored at -20°C. The variation in blood collection methods described here was due to the samples from the triploid and diploid fish originating from an independent study where plasma had been collected.

**Table 4.1.** Sea-cage cultured Atlantic salmon assessed for anti-*Neoparamoeba* spp. antibodies in the current study required multiple freshwater baths for AGD over the grow-out period. Putatively AGD-resistant broodstock were not treated for AGD for over 250 days. Triploid fish were maintained in two independent pens throughout the sampling period while the diploid fish were initially held in a single pen and were split across two cages after 10 months in sea-cage culture. These data summarise the number of months of sea-cage culture at the time of sampling, sampling month and average sea water temperature, pen allocation, number of freshwater baths administered, days elapsed post-last bath, number of sero-positive fish and the total number of fish sampled.

| Group | Months in sea-cage culture | Sampling month and average water temperature* (°C ± SE) | Pen allocation | Total Freshwater baths | Days post-last bath | Number Sero-positive/total fish sampled |
|---|---|---|---|---|---|---|
| AGD-affected Triploid | 8 | December 15.5 (0.2) | 1 | 4 | 27 | 0/10 |
| | | | 2 | 4 | 16 | 0/10 |
| | 10 | February 17.1 (0.2) | 1 | 4 | 77 | 3/10 |
| | | | 2 | 4 | 66 | 5/10 |
| | 13 | May 12.6 (0.1) | 1 | 5 | 104 | 5/10 |
| | | | 2 | 5 | 101 | 9/10 |

*Table 4.1 continued*

| Group | Months in sea-cage culture | Sampling month and average water temperature* (°C ± SE) | Pen allocation | Total Freshwater baths | Days post-last bath | Number Sero-positive/total fish sampled |
|---|---|---|---|---|---|---|
| AGD-affected Diploid | 8 | December 15.5 (0.2) | 3 | 4 | 10 | 5/20 |
| | 10 | February 17.1 (0.2) | 3 | 4 | 60 | 17/20 |
| | 13 | May 12.6 (0.1) | 3a | 5 | 95 | 4/10 |
| | | | 3b | 6 | 15 | 5/10 |
| Putative AGD-resistant broodstock | 15 | March 16.4 (0.1) | N/A | N/A | >250 | 45/55 |

* Average seawater temperature for the month of sampling taken at a depth of 3m.

**Detection of anti-*Neoparamoeba* spp. antibodies**

*Cultured and wild-type amoebae*

Wild-type amoebae were isolated as described by Morrison, Crosbie and Nowak, (2004) from AGD-affected Atlantic salmon housed at the University of Tasmania aquaculture center. As all cultured *Neoparamoeba* spp. tested to date are avirulent (Kent, Sawyer and Hedrick, 1988; Howard, Carson and Lewis, 1993; Findlay, 2001; Morrison, Crosbie, Cook, Adams and Nowak, 2005; Vincent, Adams, Crosbie, Nowak and Morrison, 2007) two previously characterised clonal strains (Dyková et al., 2005) of *Neoparamoeba* spp. were used to discriminate between reactive epitope(s) of wild-type and cultured *Neoparamoeba* spp.. These were *Neoparamoeba pemaquidensis* (NP251002) (Morrison et al., 2005) isolated from AGD-affected Atlantic salmon, and *Neoparamoeba branchiphila* (SEDMH1) isolated from sediment of Macquarie Harbour, Tasmania. Amoebae were maintained on sea water malt yeast agar; 75% (v/v) coarse-filtered sea water (35‰), 25% (v/v) distilled water, 0.01% (w/v) malt, 0.01% (w/v) yeast (Oxoid, Hampshire, England), 2% (w/v) Bacto agar (Becton, Dickson and Co., Sparks, Maryland, USA). Cells were harvested by washing the agar with sterile sea water using a transfer pipette. Wild-type and cultured cells were concentrated by centrifugation at 500× *g* for 5 min and enumerated by hemacytometer. For Western blot and ELISA assays amoebae were washed twice with phosphate buffered saline (PBS, pH 7.2) and the cell pellet was stored at -80° C.

*SDS PAGE and Western blot*

Binding of plasma (triploid and diploid) and serum (AGD-resistant) antibodies to cultured and wild-type *Neoparamoeba* spp. was assessed by Western blot. Amoebae antigens were reduced in buffer containing β-mercaptoethanol by boiling for 10 min and separated through 6% polyacrylamide gels with 8 x $10^4$ cell equivalents loaded in each lane (12.4 μg total protein/lane). Antigens were transferred to nitrocellulose membrane (Hybond-C extra, Amersham Biosciences, Little Chalfont, UK) using a semi-dry transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA) and membranes were blocked in casein solution (Vector, Burlingame, CA, USA). Blocking and antibody incubation steps were for 30 min and in between incubation steps, membranes were washed 3× 4 min with tris-buffered saline (TBS, pH 7.2). Following the final antibody incubation, membranes were washed 3× in TBS and then in 0.1M tris (pH 9.5) for 5 min. All incubation and wash steps were conducted at room temperature. Initial screening of the 175 samples was conducted using pooled serum and plasma. Pools consisting of serum or plasma from 5 to 7 fish from the same sample group were incubated at 1:100 with membrane strips (single lane) of wild-type *Neoparamoeba* spp. antigen. Bound antibodies were detected with rabbit anti-salmon IgM at 1:500 (kind gift from Dr. D. Zilberg), sheep anti-rabbit alkaline phosphatase (AP) (Chemicon, Boronia, Australia) at 1:1000 and developed with 5-bromo-4-chloro-3-indoly L phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma, St Louis, Missouri, USA) following the manufacturer's instructions. Binding of the polyclonal rabbit anti-salmon IgM to the heavy chain

of Atlantic salmon IgM has been previously described (Vincent, et al., 2006). Pooled samples that returned a positive Western blot result were subsequently screened individually as described above. In parallel, normal salmon plasma and serum pooled from 5 fish held in fresh water and therefore AGD-naïve was included as negative controls. Finally, positive samples were pooled (5 per pool) and assessed for antibody binding to cultured amoebae antigens. As the detection limit using chemiluminescence is more sensitive than BCIP/NBT, pooled positive serum and plasma were incubated at 1:500 and bound antibodies were detected with rabbit anti-salmon IgM at 1:500, sheep anti-rabbit AP at 1:2000 and enhanced chemiluminescence (ECL) using DuoLuX (Vector, Burlingame, CA, USA), Kodak BioMax Light Film and Kodak GBX developing and fixing reagents (Sigma, Castle Hills, NSW, Australia) following the manufacturer's instructions. Binding of anti-*Neoparamoeba* spp. antibodies identified here was also assessed against cultured and wild-type antigens that were separated through a 12% polyacrylamide gel to identify binding to antigens of a lower molecular weight.

*Sodium periodate oxidation of carbohydrate epitope(s) of wild-type Neoparamoeba spp.*

Wild-type *Neoparamoeba* spp. antigens were transferred to nitrocellulose membrane as outlined above and from the same membrane, bound antigens on adjacent strips were either oxidised with 20 mM sodium periodate (Merck Pty Ltd., Victoria) and 50 mM sodium borohydride (Sigma-Aldrich, St Louis, Missouri, USA) or incubated in 50 mM sodium acetate (Sigma-Aldrich) pH 4.5, following the method outlined by Woodward, Young and Bloodgood, (1985).

Membranes were then washed 3× with PBS, blocked, probed and developed by ECL as outlined above.

*Enzyme-linked immunosorbent assay (ELISA), immunocytochemistry and flow cytometry*

Binding of anti-*Neoparamoeba* spp. antibodies found in serum or plasma by Western blotting were assessed individually by ELISA. Optimal conditions for ELISA were determined empirically and have been described previously (Vincent et al., 2006). For immunocytochemistry and flow cytometry, wild-type amoebae were isolated from AGD-affected Atlantic salmon as outlined above. Wild-type amoebae were fixed in seawater Davidson's fixative (SWD) for 1 h at 20°C and washed by four cycles of re-suspending cells in PBS and concentrating cells by centrifugation at 500 × *g* for 5 min. Amoebae were then placed in the wells ($5 \times 10^3$ cells/well) of U-bottomed 96-well microplates (Sarstedt, Ingle Farm, South Australia) and blocked in 0.1% BSA-PBS for 30 min at 4°C. Cells were probed with normal Atlantic salmon serum (pooled from 5 Atlantic salmon held in fresh water and therefore was from AGD-naïve fish) or a representative pool of salmon anti-*Neoparamoeba* spp. serum (5 fish) that tested positive for anti-*Neoparamoeba* spp. antibodies by Western blot. Cells were incubated with salmon serum at 1:10 (BSA-PBS) and bound antibodies were detected with rabbit anti-salmon IgM at 1:100 and FITC-conjugated sheep anti-rabbit IgG (Chemicon, Melbourne, Australia) at 1:50. Cells were washed 3× with PBS following each antibody incubation step and photographed (Leica DC300F, Leica Microsystems, Wetzlar, Germany) using light and fluorescent microscopy. The proportion of wild-type *Neoparamoeba* spp. expressing epitope(s) to which

the serum anti-*Neoparamoeba* spp. antibodies bound was quantified by flow cytometry (Coulter Epics, Beckman Coulter, USA). A minimum of $10^4$ events were assessed per treatment and data were analysed using WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA).

**Results**

Atlantic salmon assessed in the current study for anti-*Neoparamoeba* spp. (anti-NP) antibodies were first sampled when they had been in sea-cage culture for 8 months. From the 20 triploid and 20 diploid fish sampled at this time, anti-NP antibodies were detected by Western blot in 5 samples taken from the diploid fish (Fig. 4.1). After 10 months in sea-cage culture, anti-NP antibodies were detected in samples taken from 8 of the 20 triploid fish (3 from pen 1 and 5 from pen 2). An increase in the proportion of diploid fish that developed a detectable antibody response to wild-type *Neoparamoeba* spp. was seen with 17 of the 20 diploid fish sampled testing positive for anti-NP antibodies. A further increase in the proportion of triploid Atlantic salmon with detectable anti-NP antibodies was observed after 13 months of sea-cage culture and 14 (5 from pen 1 and 9 from pen 2) of the 20 triploid fish sampled had developed an antibody response to wild-type *Neoparamoeba* spp.. In contrast, after 13 months in culture fewer positive samples from the diploid fish were identified with 9 samples (4 from pen 3a and 5 from pen 3b) testing positive for anti-NP antibodies by Western blot. The putatively resistant Atlantic salmon broodstock had been maintained at sea for 15 months at the time of sampling. Fifty-five of the 75 fish in the broodstock population were sampled and Western blot analysis identified 45 of these fish had developed an antibody response to wild-type *Neoparamoeba* spp. (Table 4.1.).

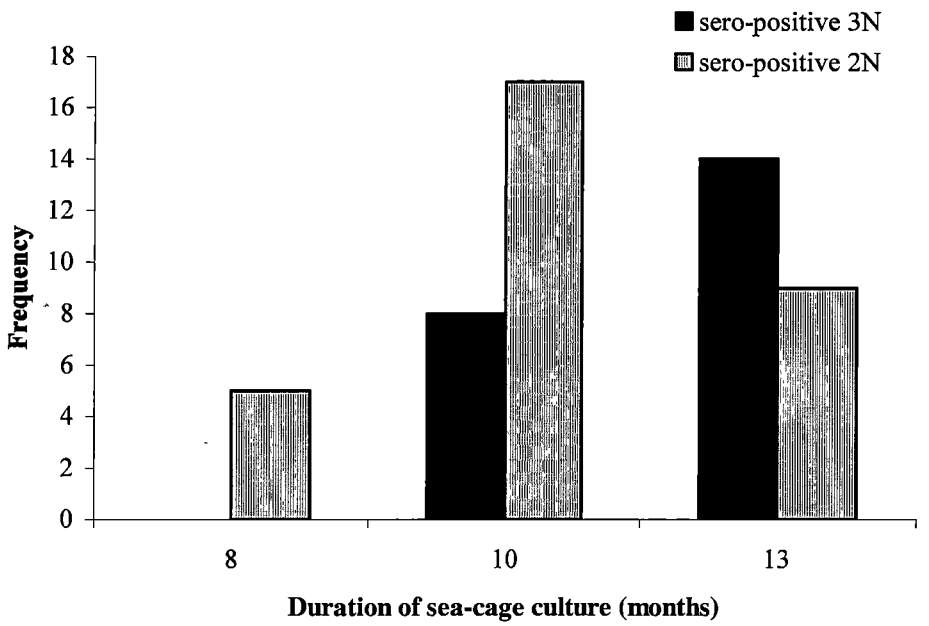


**Figure 4.1.** Antibodies that bind wild-type *Neoparamoeba* spp. were detected by Western blot in the plasma of sea-cage cultured triploid and diploid Atlantic salmon. The proportion of triploid and diploid Atlantic salmon that developed an anti-*Neoparamoeba* spp. (anti-NP) antibody response increased after 10 months in sea-cage culture. A further increase in the proportion of triploid fish with detectable anti-NP antibodies was seen after 13 months in sea-cage culture. In contrast, a decline in the proportion of diploid fish with detectable anti-NP antibodies was seen after 13 months. The frequency of sero-positive diploid (2N) and triploid fish (3N) are presented. Samples from 20 diploid and 20 triploid fish were assessed by Western blot at each sampling.

After 8 months in sea-cage culture, the gross gill assessment of the triploid and diploid fish sampled was predominantly 0 (clear-very light). The gross pathological sign of AGD was more pronounced in some fish that were sampled after 10 months in sea-cage culture. The gross gill assessment of sero-positive and sero-negative fish ranged from 0 to 3 (heavy) and the majority of sero-positive diploid fish displayed a light level of AGD with a gross gill score of 1 (Fig. 4.2a). Gross gill scores of sero-positive triploid fish from pen 1 ranged between 0 and 2 while in pen 2 scores ranged from 0 to 3. After 13 months of sea-cage culture, the majority of triploid and diploid fish assessed as having a clear to very light AGD and were assigned a gross gill score of 0 to 1. No diploid fish displayed moderate-heavy AGD as the highest gill score assigned was 1. While there was a higher proportion of sero-positive triploid fish with a gross gill score of 0, the proportion of sero-positive and sero-negative diploid fish with a gross gill score of 0 and 1 was similar. No triploid or diploid fish sampled after 13 months in sea-cage culture displayed heavy AGD infection as the highest gill score assigned was 2 (Fig 4.2b). Gross gill scores of sero-positive triploid fish from pen 1 ranged between 0 and 1 while in pen 2 scores ranges from 0 to 2. The putatively AGD-resistant broodstock had not been treated with fresh water for AGD for over 250 days and the gross gill assessment prior to sampling was recorded as clear for all fish.

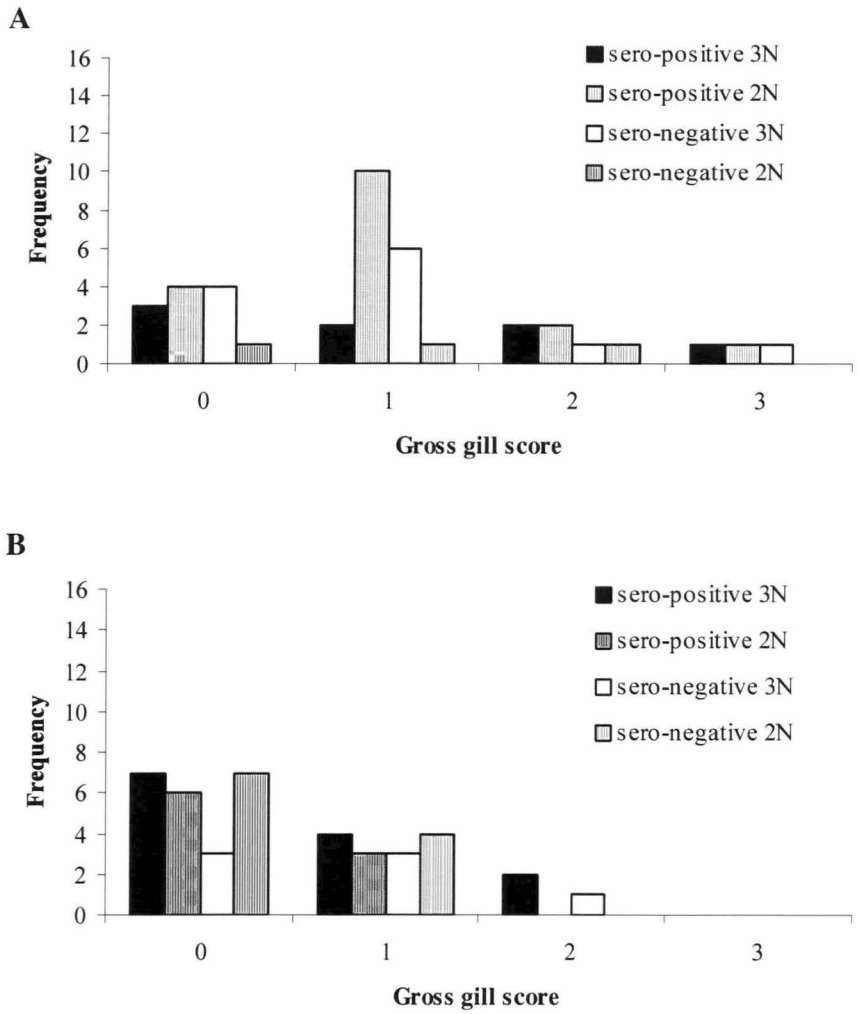


**Figure 4.2.** Assessment of AGD-like lesions on gills of Atlantic salmon from triploid and diploid populations ranged from clear to heavy in both sero-positive and sero-negative fish. After 10 months in culture (A), a greater proportion of sero-positive diploid Atlantic salmon presented with light AGD infection by gross observation. An overall higher proportion of fish presenting with clear-very light gross gill pathology was seen after 13 months in culture (B) and no fish sampled at this point were assessed with heavy AGD pathology. The frequency of sero-positive and sero-negative fish assigned the gross gill scores of 0 (clear-very light), 1 (light), 2 (moderate) and 3 (heavy) are presented. Samples from 20 diploid and 20 triploid fish were assessed at each of the 3 sampling occasions.

The Western blot profile produced by binding of plasma anti-NP antibodies present in samples from the triploid and diploid Atlantic salmon was a smear. Similarly, binding of antibodies present in serum taken from the broodstock produced a smear from around 45 kDa to > 200 kDa. Further assessment of antibody binding after antigens were separated through a 12% gel showed that below 45 kDa less anti-NP bound to the amoebae antigens and no binding was seen below 31 kDa (data not shown). As the Western blot binding profile produced by anti-NP antibodies detected in the abovementioned samples was consistent, the remaining analysis was performed using a pooled sub-group (n=5) of serum taken from the broodstock and will be from here on referred to as salmon anti-NP serum.

Salmon anti-NP antibodies did not bind cultured *Neoparamoeba* sp. antigen and no binding occurred following periodate oxidation of wild-type antigen, suggesting that these antibodies are directed towards carbohydrate epitope(s) of wild-type *Neoparamoeba* spp. (Fig. 4.3). Binding of salmon anti-NP antibodies to wild-type *Neoparamoeba* spp. was quantified by flow cytometry, producing a fluorescence intensity significantly higher than the normal serum control (Fig. 4.4). While binding was detected by Western blot and flow cytometry, salmon anti-NP serum failed to produce an optical density in excess of the normal serum control in an ELISA (data not shown).

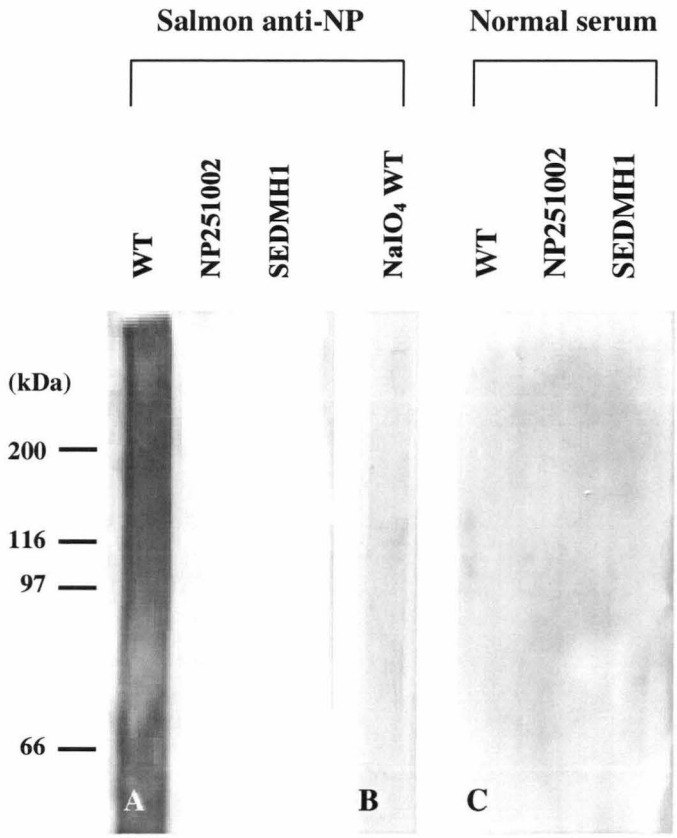


**Figure 4.3.** Binding of salmon anti-*Neoparamoeba* spp. (anti-NP) antibodies produces a smear across a broad molecular weight range (A) and reactive epitope(s) are sensitive to periodate oxidation (B). Anti-NP antibodies bind wild-type *Neoparamoeba* spp. (WT) and do not bind cultured *Neoparamoeba pemaquidensis* (NP251002) and *Neoparamoeba branchiphila* (SEDMH1) (A) and antibodies present in normal Atlantic salmon serum from AGD-naïve fish do not bind wild-type or cultured *Neoparamoeba* spp. (C). Antigens were reduced in sample buffer containing β-mercaptoethanol, separated through a 6% polyacrylamide gel and each lane was loaded with 8 x $10^4$ cell equivalents (12.4 μg total protein). Wild-type antigens were transferred to nitrocellulose and treated with sodium periodate ($NaIO_4$WT) (B). Membranes were probed with pooled (5 fish) salmon anti-NP (A) and normal Atlantic salmon serum (C). Bound antibodies were detected with rabbit anti-salmon IgM, AP-conjugated sheep anti-rabbit IgG and chemiluminescence. Normal serum was taken from Atlantic salmon held in fresh water and the fish were therefore AGD-naïve.

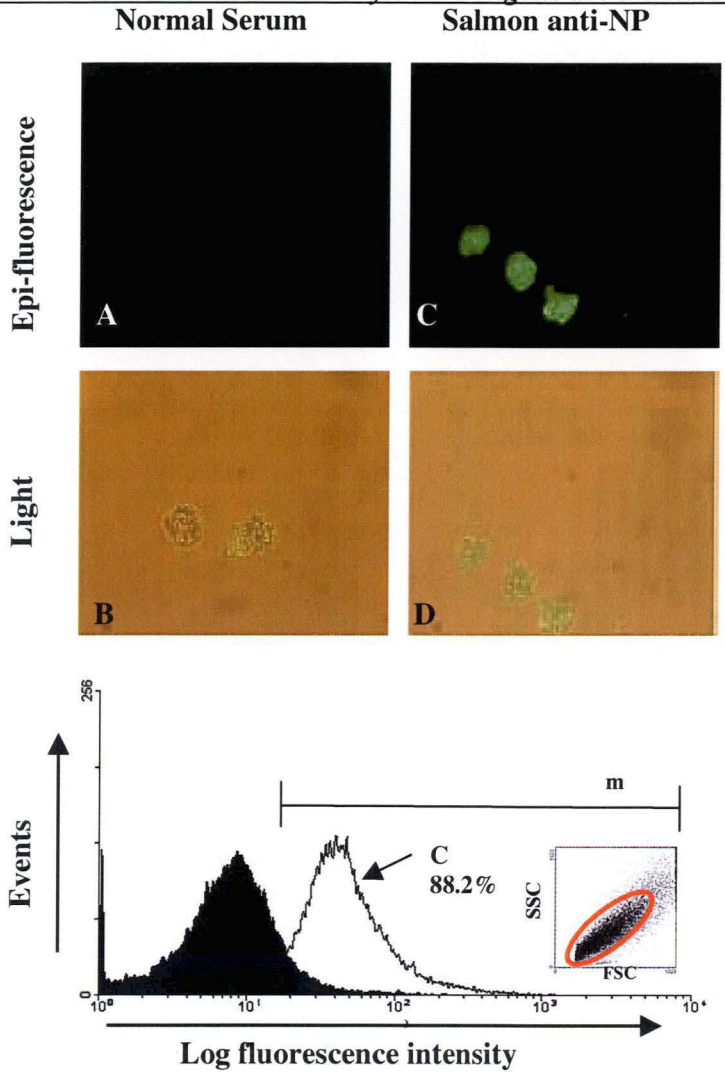


**Figure 4.4.** Serum anti-*Neoparamoeba* spp. (anti-NP) antibodies bind cell-surface epitope(s) of wild-type *Neoparamoeba* spp.. The light micrographs correspond to the adjacent epi-fluorescent images. A sub-sample of cells probed with either anti-NP of normal sera were photographed before quantitative analysis of the remaining sample by flow cytometry. Wild-type *Neoparamoeba* spp. were fixed and probed with normal salmon serum (Normal serum) or anti-NP serum (Salmon anti-NP). Bound antibodies were detected with rabbit anti-salmon IgM and FITC-conjugated sheep anti-rabbit IgG. Normal serum and salmon anti-NP serum was pooled from 5 fish. Normal serum was taken from Atlantic salmon held in fresh water and these fish were therefore AGD-naïve. Data are representative of cells within the gated region shown in the dot-plot (inset). The proportion of cells producing a fluorescence intensity significantly higher than the normal serum control (A) are presented on the histogram and represent data assessed within the marked region (m). Flow cytometric data were analysed and presented using WinMDI 2.8 software.

**Discussion**

In some cases, sea-farmed Atlantic salmon develop an antibody response to wild-type *Neoparamoeba* spp. and anti-NP antibodies detected in the current study are directed towards cell-surface carbohydrate epitope(s). Antibodies that bind cell-surface carbohydrate epitope(s) of wild-type *Neoparamoeba* spp. have also been detected in some Atlantic salmon experimentally inoculated with wild-type *Neoparamoeba* spp. (Vincent, et al., 2006; Vincent, et al., 2008). In addition, monoclonal antibodies produced against cell-surface antigens of wild-type *Neoparamoeba* spp. are predominantly directed towards carbohydrate epitope(s) (Villavedra, Lemke, To, Broady, Wallach and Raison, 2007). Together, these results suggest that cell-surface carbohydrate epitopes of wild-type *Neoparamoeba* spp. are immunodominant. Carbohydrate antigens are abundant on the cell-surface of many protozoan parasites and some of these structures are important for attachment to the host (Mendonca-Previato, Todeschini, Heise and Previato, 2005). While the mechanism(s) that mediate attachment of *Neoparamoeba* spp. to Atlantic salmon gill tissues are unknown, the dominance of cell-surface carbohydrate epitope(s) suggests that attachment may be mediated by cell-surface glycoconjugates.

While anti-NP antibodies have been detected in many fish, both in the current and previous studies (Vincent, et al., 2006; Vincent, et al., 2008), antibody titre in the majority of cases, was not detectable in an ELISA. The same Western blot binding profile (smear) as reported in the current study was produced by binding of anti-NP antibodies present in serum of some Atlantic salmon that were

exposed to wild-type *Neoparamoeba* spp. twice (Vincent, et al., 2006) and anti-NP antibodies present in the serum of 4 fish that were exposed to wild-type *Neoparamoeba* spp. for a prolonged period (Vincent, et al., 2008). Despite producing similar Western blot binding profiles, a significant serum anti-NP antibody titre was only measured by an ELISA in one of these samples (Vincent, et al., 2008). This suggests that failure to measure antibody titre by an ELISA in the current study or the study by Vincent et al., (2006) is not associated with the ELISA conditions but rather with very low antibody levels.

Wild-type *Neoparamoeba* spp. are occasionally entrapped in interlamellar vesicles containing immune-like cells (Adams and Nowak, 2001) and antigen processing may be facilitated by MHC $II^+$ cells present in AGD gill lesions (Morrison, Koppang, Hordvik and Nowak, 2006). The level of antigen processing may therefore be restricted by the number of *Neoparamoeba* spp. that become entrapped, perhaps influencing serum anti-NP antibody titre. Results presented here suggest that the presence of low-level plasma anti-NP antibodies does not appear to be related to the level of gross gill pathology. After 10 months of sea-cage culture there was a substantially higher proportion of sero-positive diploid fish with a gross gill score of 1, however sero-positive and sero-negative fish from both triploid and diploid populations were represented across the range of gross gill pathology with scores from 0 to 3. An overall reduction in gill pathology was seen after 13 months with no fish displaying heavy AGD and although no sero-positive diploid fish were assessed higher than a gill score of 1, the proportion of sero-negative and sero-positive diploid fish with gill scores of 0 and 1 were similar. There was a slight variation in the level of gross pathology of

the sero-positive fish between triploid pens 1 and 2 and the diploid pens 3a and 3b however little can be concluded from this due to the low sample size.

The number of fish that developed an anti-NP response increased over time with the exception of the diploid fish at the final sampling after 13 months in culture. As only 10 fish were sampled from each cage, the decline in the number of sero-positive diploid fish identified at the final sampling may have been influenced by the low numbers of fish sampled. It has been hypothesised that the duration of, or multiple exposure to, wild-type *Neoparamoeba* spp. may influence the development of an antibody response in Atlantic salmon (Vincent, et al., 2006; Vincent, et al., 2008). The development of a serum antibody response to the ectoparasitic copepod, *Lepeophtheirus salmonis,* appears to be influenced by the duration of exposure and/or parasite abundance. Rainbow trout naturally exposed to a low-level *L. salmonis* infection for 8 weeks (Grayson, Jenkins, Wrathmell and Harris, 1991) and Atlantic salmon exposed to a moderate *Caligus elongatus* infection for 12 months do not develop a serum antibody response (MacKinnon, 1993). While Atlantic salmon exposed to a high-level *L. salmonis* infection for up to 2 years develop a serum antibody response (Grayson, et al., 1991). Similarly for AGD-affected Atlantic salmon, the level of infection and potentially, the subsequent increase in antigen processing events may influence the development of a serum antibody response in AGD-affected Atlantic salmon. While data presented here provide some support for this, the same fish weren't assessed over time. Therefore any relationship between the length of time fish were exposed to wild-type *Neoparamoeba* spp., or the time post last bath, and the development of a serum antibody response cannot be drawn from results

presented in this study. Furthermore, there is no relationship between the level of gill pathology and presence of serum antibodies.

The time elapsed between freshwater bath treatments increased over time in culture with the exception of one pen of diploid fish. An increase in the period between freshwater bathing for AGD may be interpreted as an indication of resistance. However, environmental conditions including increased salinity and temperature are key factors that influence AGD (Clark and Nowak, 1999; Adams and Nowak, 2003; Adams and Nowak, 2004b). The greatest period between freshwater bath treatments occurred between the second and third sampling that coincided with the period between late summer and late autumn where a seasonal reduction in seawater temperature occurred. In addition, it is common practice during marine culture of salmon to move sea cages to allow for fallowing of the site and to facilitate fresh water bathing. The Atlantic salmon sampled in the current study were held in cages that were routinely moved around the sites for fresh water bathing. It is therefore possible that fish were exposed to different levels/timing of infection, however due to the low sample size and the fact that individual fish were not sampled over time, no relationship between the sero-positive fish (either diploid or triploid) and the level of infection or length of time exposed can be drawn.

Freshwater bathing is conducted on the basis of gross gill pathology and gill score data collected after 13 months in sea-cage culture suggests that at this time fish were experiencing a lower level of AGD. The putatively AGD-resistant

Atlantic salmon broodstock did not require a freshwater bath for over 8 months yet the development of a serum antibody response in a large proportion of these fish indicates that they were exposed to *Neoparamoeba* spp.. These fish were housed in a single sea-cage at a very low stocking density and this may have attributed to the low-level of infection Alternatively, as these Atlantic salmon were the first progeny of broodstock that were previously selected by HAC on the basis of gross gill pathology, resistance to AGD in terms of gross gill pathology may be inherent.

There are many challenges associated with the development of anti-parasite vaccines and this is reflected by the few anti-parasite vaccines commercially available. Vaccines for the cattle tick, *Boophilus microplus*, were introduced in 1994 and are currently the only commercially available ectoparasitic vaccines (Nuttall, Trimmel, Kazimirova and Labuda, 2006). Identification of protective peptide antigens is important for the development of an economically viable AGD vaccine. To date, reactivity of salmon anti-NP antibodies to putative peptide epitope(s) remains an isolated case (Vincent, et al., 2008) and carbohydrate epitope(s) are predominantly recognised by Atlantic salmon serum anti-NP antibodies. In addition, monoclonal antibodies produced against deglycosylated wild-type cell-surface antigens react with very few cell-surface peptide epitope(s) that are unique to wild-type *Neoparamoeba* spp. (Villavedra, et al., 2007). Together, this suggests that the identification of peptide candidate vaccine antigens by screening serum against wild-type *Neoparamoeba* spp. is unlikely. Mucosal antibodies have been associated with resistance of fish against protozoan parasites such as *I. multifiliis* (Wang and Dickerson, 2002; Xu and

Klesius, 2002) and *Cryptocaryon irritans* (see Luo, et al., 2006; Yambot and Song, 2006). While anti-NP antibodies were not detected in cutaneous mucus of putatively AGD-resistant Atlantic salmon (Vincent, et al., 2006), resistance of Atlantic salmon to *Neoparamoeba* spp. may be associated with a more localised response. The potential that a more localised antibody response, in the gill mucus or epithelium, may play a role in resistance of Atlantic salmon to AGD warrants further investigation.

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# Chapter 5

# Cutaneous mucus and saccharides reduce the ability of wild-type *Neoparamoeba* spp. to elicit amoebic gill disease (AGD) in Atlantic salmon

**Abstract**

Attachment of parasites to host tissues is in many cases mediated by parasite cell-surface lectins. Wild-type *Neoparamoeba* spp. were incubated in Atlantic salmon, *Salmo salar* L., cutaneous mucus or a range of saccharides prior to inoculation of sea water systems containing amoebic gill disease (AGD)-naïve Atlantic salmon. The ability of wild-type *Neoparamoeba* spp. to elicit AGD was significantly reduced by Atlantic salmon cutaneous mucus and all saccharide treatments including galactose and GalNAc. It was therefore hypothesised that wild-type *Neoparamoeba* spp. may express an orthologue of the *Entamoeba histolytica* Gal/GalNAc inhibitable lectin. Antibodies that bind the Gal/GalNAc inhibitable lectin of *E. histolytica* were assessed for their ability to bind cell-surface antigen(s) of wild-type *Neoparamoeba* spp.. These antibodies bound live wild-type *Neoparamoeba* spp., yet glucose or galactose enhanced, rather than inhibited antibody binding. Results presented here suggest that the ability of wild-type *Neoparamoeba* spp. to elicit AGD may be associated with lectin-mediated attachment.

## Introduction

*Neoparamoeba perurans* is the agent of amoebic gill disease (AGD) in sea-farmed Atlantic salmon, *Salmo salar* L., in southern Tasmania, Australia (Young, Crosbie, Adams, Nowak and Morrison, 2007). Gill-derived *Neoparamoeba* are isolated from the gill tissues of AGD-affected Atlantic salmon by plastic adherance (Morrison, Crosbie and Nowak, 2004). *N. pemaquidensis* and *N. branchiphila* have also been isolated by culture from gill tissues of AGD-affected Atlantic salmon (Dyková, Nowak, Crosbie, Fiala, Pecková, Adams, Macháčková and Dvořáková, 2005). Therefore, amoebae obtained from gill tissues of AGD-affected Atlantic salmon are referred to as wild-type *Neoparamoeba* spp.. AGD is a global health concern for Atlantic salmon growers and has been reported in Ireland (Rodger and McArdle, 1996; Palmer, Carson, Ruttledge, Drinan and Wagner, 1997), the USA (Kent, Sawyer and Hedrick, 1988), Chile, New Zealand, Australia (Munday, 1986; Munday, Zilberg and Findlay, 2001) Scotland (Young, Dyková, Snekvik, Nowak and Morrison, 2008) and Norway (Steinum, Kvellestad, Rønneberg, Nilsen, Asheim, Fjell, Nygård, Olsen and Dale, 2008). AGD is the greatest health concern of Atlantic salmon growers in southern Tasmania, Australia and to date, freshwater bathing is the only commercially applied treatment for AGD. In southern Tasmania, the prevalence of AGD increases during summer months in association with warmer sea water temperatures and salinity of 35‰ (Clark and Nowak, 1999; Adams and Nowak, 2003) and during this period, fish require repetitive freshwater baths. Freshwater bathing is labor and cost-intensive, therefore the development of an alternative

measure for alleviating AGD, such as an anti-AGD vaccine, is a priority for salmon growers in Tasmania.

Attachment of a range of parasites to host cells, in some cases, may be mediated by the binding of parasite lectins to carbohydrate residues of host tissues. In many instances, these interactions can be inhibited by blocking the carbohydrate recognition domain (CRD) of the lectin with target saccharides, host tissues or with antibodies that bind the CRD. For example, attachment of *Giardia lamblia* trophozoites to Caco-2 cells is mediated by a mannose binding lectin (Katelaris, Naeem and Farthing, 1995) and inhibited by mucin (Roskens and Erlandsen, 2002). *Acanthamoeba castellanii* attachment *in vitro* to corneal epithelial cells is mediated by a mannose binding lectin and this interaction is inhibited by mannose (Yang, Cao and Panjwani, 1997; Cao, Jefferson and Panjwani, 1998). The human pathogenic amoebae, *Entamoeba histolytica,* utilise the Gal/GalNAc inhibitable lectin for attachment to colonic mucins (Ravdin and Guerrant, 1981; Ravdin, John, Johnston, Innes and Guerrant, 1985; Chadee, Petri, Innes and Ravdin, 1987). Either monoclonal antibodies that bind the *E. histolytica* Gal/GalNAc inhibitable lectin, galactose or GalNAc block lectin-mediated attachment of *E. histolytica* to target carbohydrate residues (Ravdin, Petri, Murphy and Smith, 1986; Venkataraman, Haack, Bondada and Kwaik, 1997; Kwaik, Venkataraman, Harb and Gao, 1998). The Gal/GalNAc inhibitable lectin has shown potential as a vaccine antigen against amebiasis in animal models (Zhang, Cieslak and Stanley, 1994; Soong, Kain, Abd-Alla, Jackson and Ravdin,

1995; Dodson, Lenkowski, Eubanks, Jackson, Napodano, Lyerly, Lockhart, Mann and Petri, 1999). *Hartmannella vermiformis* also express an orthologue of the Gal/GalNAc inhibitable lectin (Venkataraman, et al., 1997) and *H. vermiformis* are ancestrally related to *N. pemaquidensis* and *N. aestuarina* (Peglar, Amaral Zettler, Anderson, Nerad, Gillevet, Mullen, Frasca, Silberman, O'Kelly and Sogin, 2003). It was therefore hypothesised that wild-type *Neoparamoeba* spp. may also express an orthologue of the Gal/GalNAc inhibitable lectin.

In the current study, wild-type *Neoparamoeba* spp. were incubated in a range of saccharides or Atlantic salmon cutaneous mucus prior to induction of AGD. In addition, monoclonal and polyclonal antibodies that bind the Gal/GalNAc inhibitable lectin of *E. histolytica* were assessed for their ability to bind antigen(s) of wild-type *Neoparamoeba* spp.. Incubation of wild-type *Neoparamoeba* spp. with either Atlantic salmon cutaneous mucus or each of the saccharides assessed, significantly reduced the ability of these amoeba to elicit AGD in naïve Atlantic salmon. Both the monoclonal and polyclonal anti-Gal/GalNAc inhibitable lectin antibodies bound live wild-type *Neoparamoeba* spp., yet binding was not inhibited by galactose.

## Materials and methods

### Induction of AGD in Atlantic salmon by inoculation with wild-type *Neoparamoeba* spp. pre-treated with carbohydrate preparations.

*Amoebae preparations*

Wild-type *Neoparamoeba* spp. were isolated from AGD-affected Atlantic salmon housed at the University of Tasmania Aquaculture Centre as described by Morrison et al., (2004). Wild-type *Neoparamoeba* spp. were incubated with a range of saccharides, Atlantic salmon cutaneous mucus or PBS prior to the inoculation of fish-holding systems (Table 5.1.). Mucus was obtained from AGD-naïve Atlantic salmon that were acclimitised to sea water (1 μm-filtered) and had not been exposed to wild-type *Neoparamoeba* spp.. Fish were anaesthetised with Aqui-S (Aqui-S NZ Ltd, Lower Hutt, New Zealand) and mucus was collected by gently scraping the skin with the edge of a glass slide. Anti-protease cocktail (Sigma) was added to the mucus suspension according to the manufacturer's instructions. Mucus was then vortexed, spun at 16 000 ×*g* for 15 min and the supernatant removed. The mucus supernatant was 0.2 μm-filtered, concentrated and dialysed against PBS using a 10 kDa molecular weight cut-off filtration column (Amicon Ultra, Millipore, Carrigtwohill, County Cork, Ireland) following the manufacturer's instructions. The total protein concentration of the mucus suspension was quantified by a colorimetric assay (Pierce, Rockford, USA). Wild-type *Neoparamoeba* spp. were exposed to each of the saccharides (all purchased from Sigma), Atlantic salmon cutaneous mucus supernatant or PBS alone (Table 5.1.). For each of the treatments, wild-type

*Neoparamoeba* spp. were incubated in a volume of 1 mL at 4°C for 20 min. Cells were washed 1 × in PBS, concentrated and resuspended in 0.2 µm-filtered sea water. Cells were then directly transferred to experimental systems within 10 min of re-suspension. To assess the viability of cells after treatment, an aliquot of 10 µL was taken from each treatment, placed on a glass slide and overlayed with 200 µL 0.2 µm-filtered sea water (35‰). After 15 min, cell viability was confirmed after observing cell adherence to the glass slide.

**Table 5.1.** Summary of the saccharides and inoculating concentration of wild-type *Neoparamoeba* spp. used for the induction of AGD. Wild-type *Neoparamoeba* spp. were exposed to the following treatments in PBS for 20 min at 4°C. Cells were then re-suspended in sterile sea water and directly transferred to experimental systems. All saccharides were diluted in PBS and the mucus supernatant was dialysed against PBS.

| **Trial and concentration of inoculum (cells/L)** | **Treatment** |
|---|---|
| 1 (8500) | PBS |
| | Galactose (500mM) |
| | Glucose (500mM) |
| | GalNAc (50mM) |
| | Atlantic salmon cutaneous mucus supernatant* |
| 2 (8500) | PBS |
| | Mannose (500mM) |
| | Fucose (500mM) |
| | Xylose (500mM) |
| | Atlantic salmon cutaneous mucus supernatant* |

*540 µg/mL total protein. The mucus supernatant used in trial 1 and 2 was taken from the same preparation.

*Fish and experimental conditions*

Atlantic salmon (100-150 g) were obtained from the Saltas hatchery, Wayatinah, Tasmania. These fish had only been maintained in fresh water and therefore had not been exposed to wild-type *Neoparamoeba* spp.. Fish were held in a single 3000 L recirculation system and acclimated to 35‰ salinity by multiple sea water (1 µm-filtered) exchanges over a 10 d period. For exposure to wild-type *Neoparamoeba* spp., groups of 5 fish were transferred to 80 L tanks containing 40 L sea water (35 ‰ at 16.5°C) with aeration. Two tanks were used for each treatment (10 fish/treatment). A further 2 groups of 5 fish were maintained under the same conditions but were not exposed to wild-type *Neoparamoeba* spp.. Two independent infection trials were conducted. Positive (wild-type *Neoparamoeba* spp. incubated in PBS alone) and negative (no amoebae) infection controls were included in each trial. In trial 1 and 2, the mucus supernatant treatment was replicated using mucus from the same preparation. Both trials were conducted according to the protocol developed by Crosbie, Adams, Attard and Nowak, (2007) with one minor alteration. After the initial 6 h exposure to wild-type *Neoparamoeba* spp., each treatment group of 10 fish (2 tanks of 5 fish each) was transferred to individual 350 L recirculating systems (35 ‰ at 16.5°C) for 3 d. During the 3 d, water changes of approximately 30% of the system volume were conducted for each treatment system after 24 and 48 h and at all times the total ammonia levels were 0.25-1.5 mg/L. Fish were not fed during the experimental period. Mortality not associated with AGD, or loss due to fish jumping out of

tanks was experienced in some experimental systems in both trial 1 and 2. Therefore, 8 fish were randomly sampled from each experimental system at the end of the 3 d trial.

*Assessment of gill pathology*

Fish were terminally anaesthetised with Aqui-S and the entire gill basket was excised and placed in seawater Davidson's fixative (SWD). The second, left gill arch was processed and embedded following routine histological protocols and sections (5 μm) were stained with haematoxylin and eosin (H and E). Gill sections were assessed by light microscopy at 400× magnification. Gill lesions were designated as AGD related when amoebae trophozoites with visible nucleus and *Perkinsiella* amoebae-like organisms (PLOs) (Dyková et al., 2005) were seen in association with hyperplastic tissues (Adams and Nowak, 2001). Filaments were included in the assessment of the number of AGD lesions when the central venous sinus was visible in at least two-thirds of the filament length (Adams and Nowak 2003), a minimum of 10 gill filaments per section were assessed.

*Data analysis*

Differences in gill pathology between treatment groups were assessed by analysis of variance. Data were initially tested for homogeneity of variances using Levene's test. Means were compared by Tukey's HSD test and due to non-homogeneous variances, differences were considered significant at $P < 0.01$.

Data were analysed using SPSS statistical software (Version 13.0, SPSS Science, Chicago, IL,USA).

**Binding of anti-Gal/GalNAc inhibitable lectin antibodies to wild-type *Neoparamoeba* spp..**

*SDS-PAGE and Western blot*

Amoebae antigens were reduced by boiling for 10 min in buffer containing β-mercaptoethanol and 8 x $10^4$ cell equivalents/lane were separated through 6% polyacrylamide gels. Antigens were transferred to nitrocellulose membranes (Hybond-C extra, Amersham Biosciences, Little Chalfont, UK) using a semi-dry transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA) and membranes were blocked in casein solution (Vector, Burlingame, CA, USA). Blocking and antibody incubation steps were for 30 min and in between incubation steps, membranes were washed 3 × 4 min with tris-buffered saline (TBS, pH 7.2). Following the final antibody incubation, membranes were washed 3 × in TBS and then in 100 mM tris (pH 9.5) for 5 min. All incubation and wash steps were conducted at 20°C. Membranes were probed with Staphylococcal protein A-purified monoclonal (3F4, 100 μg/mL) or rabbit polyclonal ("Shiro", 0.6 μg/mL) antibodies that bind the Gal/GalNAc inhibitable lectin of *Entamoeba histolytica* (a kind gift from Prof. William A. Petri Jr.). Mouse IgG (100 μg/mL) or rabbit IgG (0.6 μg/ml) were used as negative controls. All antibodies were diluted in casein solution. Bound antibodies were detected with alkaline phosphatase (AP)-conjugated goat anti-mouse (Sigma) or AP-conjugated sheep anti-rabbit IgG (Chemicon, Boronia, Australia) at 1:1000 and enhanced

chemiluminescence (ECL) using DuoLuX (Vector, Burlingame, CA, USA), Kodak BioMax Light Film and Kodak GBX developing and fixing reagents (Sigma, Castle Hills, NSW, Australia) following the manufacturer's instructions.

*Immunocytochemistry and flow cytometry*

Binding of 3F4 or Shiro to live and SWD fixed wild-type *Neoparamoeba* spp. was assessed. Wild-type *Neoparamoeba* spp. were isolated as outlined above. Cells were fixed in SWD for 20 min at 20°C and washed 3 × with PBS. Live cells were harvested, re-suspended in 5 mL of 0.2 μm-filtered sea water and maintained in suspension by gentle rocking. Prior to incubation with antibodies, all cells were washed 1 × in PBS. Amoebae were placed in wells ($5 \times 10^4$ cells/well) of U-bottomed 96-well microplates (Sarstedt, Ingle Farm, South Australia) and incubated in 50 μL Shiro or rabbit IgG isotype control (Sigma, St Louis, MO, USA) at 0.6 μg/mL or 3F4 or monoclonal IgG isotype control (Sigma) at 100 μg/mL for 20 min at 4°C. All antibodies were diluted in 0.1% BSA-PBS. After incubation with primary antibodies, cells were washed 2 × in PBS. Live cells were then fixed in SWD for 20 min at RT and washed 3 × with PBS. Cells that were fixed before incubation with primary antibodies were washed 2 × with PBS. The binding of Shiro or purified rabbit IgG to wild-type *Neoparamoeba* spp. was detected with FITC-conjugated sheep anti-rabbit IgG (Chemicon, Boronia, Australia) and binding of 3F4 or purified murine IgG antibodies was detected with FITC-conjugated goat anti-mouse IgG (Sigma) following the manufacturer's instructions. Cells were washed a further 2 × in

PBS. The effect of galactose or glucose on the binding of 3F4 to live wild-type *Neoparamoeba* spp. was assessed by incubating cells in 500 mM galactose or glucose (Sigma) in PBS for 20 min at 4°C. Wild-type *Neoparamoeba* spp. were then washed 1 × in PBS, probed with 3F4 and FITC-conjugated goat anti-mouse IgG as outlined above. A sub-sample of cells from each treatment were photographed (Leica DC300F, Leica Microsystems, Wetzlar, Germany) using light and epi-fluorescence microscopy. The proportion of wild-type *Neoparamoeba* spp. expressing epitope(s) to which the anti-Gal/GalNAc inhibitable lectin antibodies bound was quantified using flow cytometry (Coulter Epics, Beckman Coulter, USA). Ten thousand cells were assessed per treatment and data were analysed using WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA).

## Results

### Induction of AGD in Atlantic salmon by inoculation with wild-type *Neoparamoeba* spp. pre-treated with carbohydrate preparations.

Wild-type *Neoparamoeba* spp. may colonise Atlantic salmon gill tissues via lectin-mediated attachment. To assess this hypothesis, wild-type *Neoparamoeba* spp. were incubated in a range of saccharides or Atlantic salmon cutaneous mucus supernatant before inoculating fish-holding tanks containing AGD-naïve Atlantic salmon. After incubation in suspension with each of the saccharides or mucus supernatant, wild-type *Neoparamoeba* spp. from all treatments of the 2 independent trials adhered to glass confirming their viability (Fig. 5.1A). Cell nuclei and *Perkinsiella* amoebae-like organisms (PLOs) were visible in many

adhered cells (Fig. 5.1B) and mitotic division of amoebae was observed (Fig. 5.1C). No agglutination of *Neoparamoeba* spp. was observed after incubation in mucus supernatant or any of the saccharides assessed.

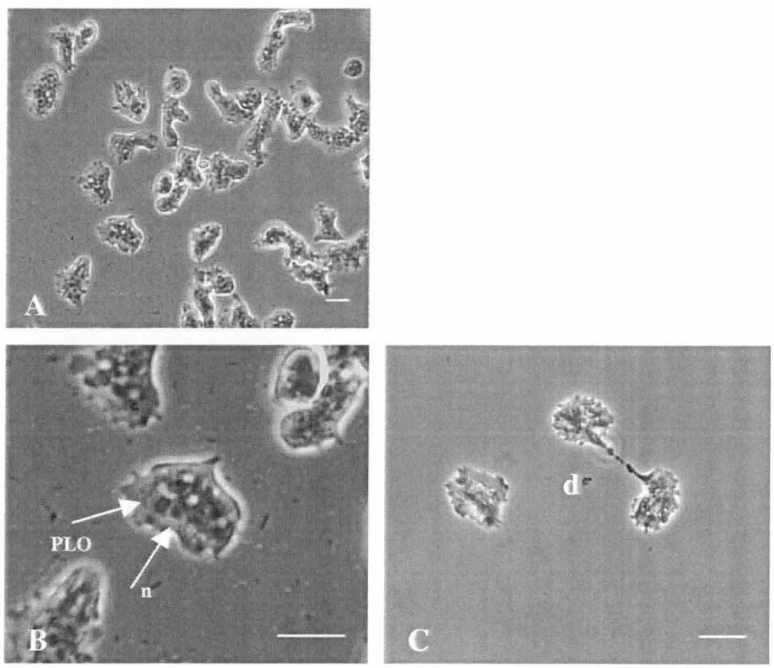


**Figure 5.1.** The viability of wild-type *Neoparamoeba* spp. was not affected after incubation with mucus supernatant or saccharide preparations. Trophozoites readily adhered to glass slides within 15 min (A). The above images of cells after treatment with 500 mM galactose is representative of wild-type *Neoparamoeba* spp. cell adherence after exposure to all saccharide treatments, Atlantic salmon cutaneous mucus supernatant or PBS alone. The cell nuclei (n) and Perkinsiella amoebae-like organisms (PLO) were easily recognised in adhered cells (B) and cells undergoing mitotic division (d) were seen (C). Scale bars = 40 μm.

AGD lesions were present on gills of all fish exposed to wild-type *Neoparamoeba* spp. (Fig. 5.2). Wild-type *Neoparamoeba* spp. with visible cell nuclei and PLOs were seen in association with lesions formed by hyperplasia of gill epithelium (Fig. 5.2E). AGD lesions did not develop in the gills of fish that were not exposed to wild-type *Neoparamoeba* spp. and normal gill structure was observed (data not shown). In both trials there was a significant reduction in the number of AGD lesions that developed across all treatments in comparison with the positive infection control (wild-type *Neoparamoeba* spp. incubated in PBS before inoculation). In trial 1 there was a significant reduction ($P = 0.001$) in the average number of AGD lesions/filament in comparison to the infection control after wild-type *Neoparamoeba* spp. was incubated with glucose, galactose, GalNAc or mucus supernatant prior to inoculating systems (Fig. 5.3A). There was no significant difference ($P = 0.597$) between the saccharide treatments in trial 1. Similarly, incubation of wild-type *Neoparamoeba* spp. in mannose, xylose, fucose or mucus supernatant prior to inducing AGD significantly reduced ($P = 0.000$) the development of AGD pathology in trial 2 (Fig. 5.3B). Treatments applied in trial 2 included mannose, fucose, xylose and mucus supernatant. All trial 2 treatments were equally effective in reducing the development of AGD lesions ($P = 0.541$). The Atlantic salmon cutaneous mucus supernatant treatment was included in both trial 1 and trial 2 and was obtained from AGD-naïve Atlantic salmon. The mucus supernatant used in trial 1 and trial 2 was from the same preparation. The number of AGD lesions on fish exposed wild-type

*Neoparamoeba* spp. incubated in mucus supernatant were 58.8% and 71.7% lower than the corresponding infection control in trial 1 and 2 respectively.

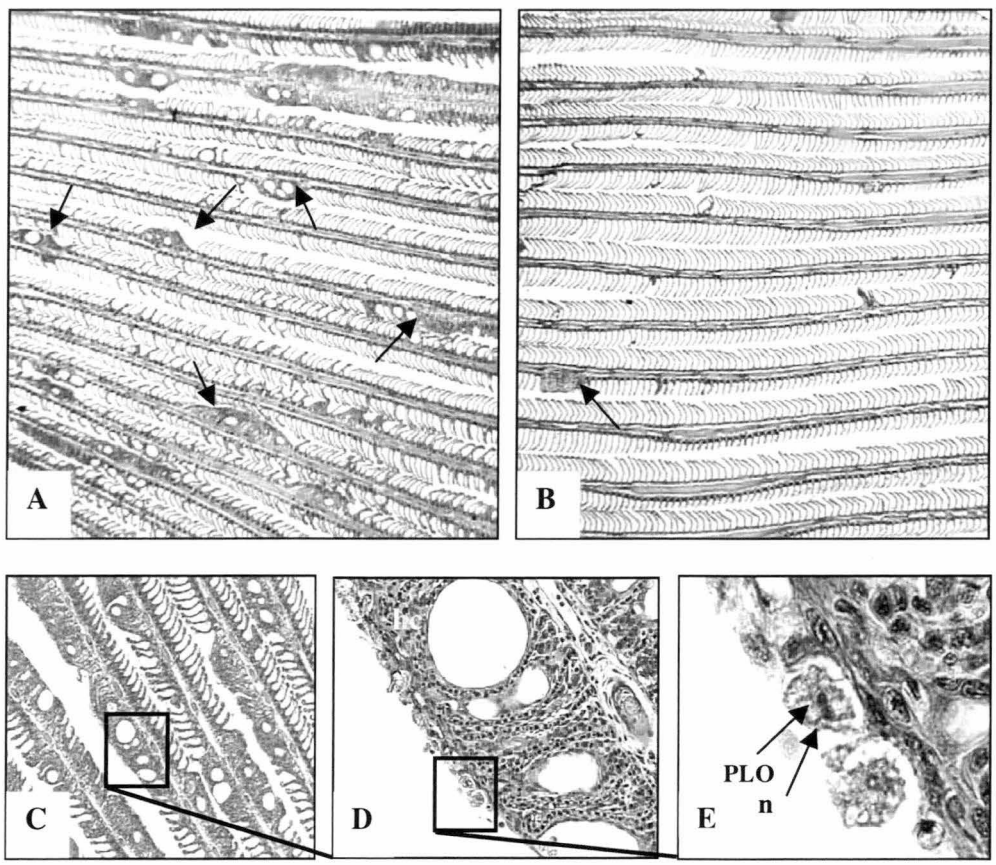


**Figure 5.2.** Atlantic salmon exposed to a high-density inoculum of wild-type *Neoparamoeba* spp. for 6 h develop AGD lesions (arrows) within 3 d. Very few AGD lesions were found on the majority of fish that were exposed to wild-type *Neoparamoeba* spp. that were pre-incubated in saccharide or mucus treatments (B). The number of AGD lesions was substantially higher on gills of fish that were exposed to wild-type *Neoparamoeba* spp. that were treated with PBS alone (A). Areas of hyperplastic cells (hc) associated with trophozoites were confirmed to be AGD-lesions after observation of the nucleus (n) and Perkinsiella amoebae-like organism (PLO) in lesion associated trophozoites. Images C-E show the sequential enlargement of an AGD lesion that developed on the gills of a fish exposed to the infection control inoculum (*Neoparamoeba* spp. incubated in PBS). The above images are representative of gill pathology of fish exposed to the infection control inoculum of wild-type *Neoparamoeba* spp. (A) or any of the saccharide or mucus supernatant preparations (B) listed in Table 5.1.

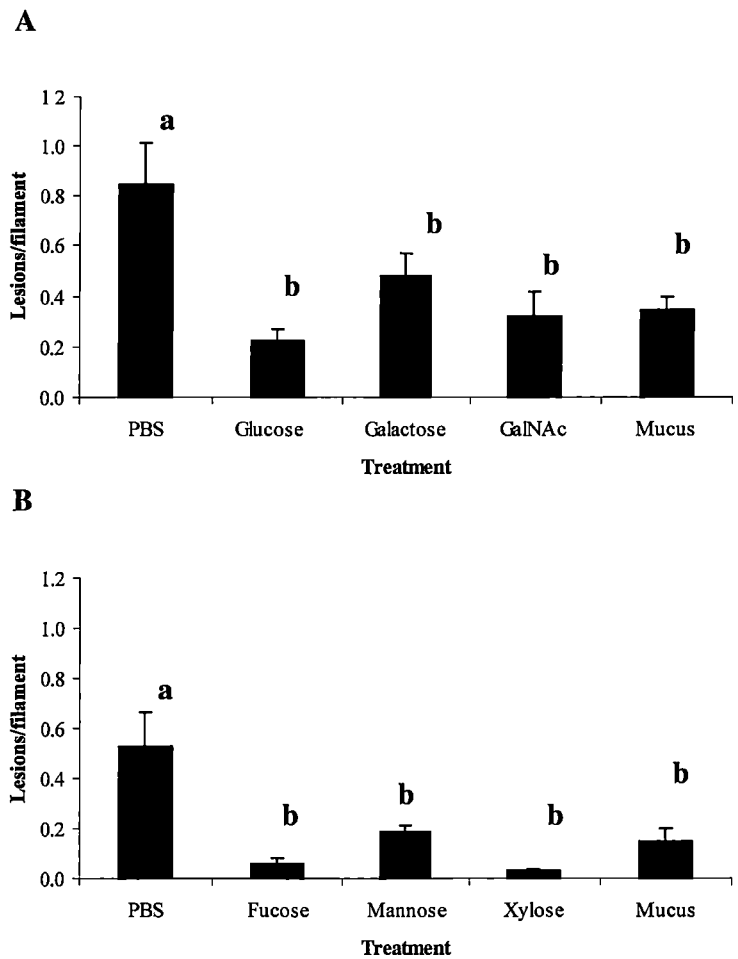


**Figure 5.3.** Incubation of wild-type *Neoparamoeba* spp. with a range of saccharides or mucus supernatant prior to inoculating fish-holding systems significantly (P = 0.000) reduced the number of AGD lesions that developed on the gills of exposed Atlantic salmon. These data are from 2 independent trials. In trial 1, wild-type *Neoparamoeba* spp. were incubated in glucose or galactose at 500 mM in PBS, GalNAc-PBS at 50 mM, Atlantic salmon cutaneous mucus supernatant (540 µg/mL total protein) or PBS alone (A). In trial 2, wild-type *Neoparamoeba* spp. were incubated in fucose, mannose or xylose at 500 mM in PBS, Atlantic salmon cutaneous mucus supernatant (540 µg/mL total protein) or PBS alone (B). Atlantic salmon mucus was collected from AGD-naïve fish maintained in 35 ‰ sea water. The supernatant was concentrated by centrifugation (10 kDa MW filtration unit) and dialysed against PBS. Tanks were inoculated at $8.5 \times 10^3$ cells/L and Atlantic salmon were exposed to these conditions for 6 h. Fish were then transferred to independent systems for 3 d. The average number of lesions/filament is presented (± SEM, n=8 fish) for each treatment group.

**Binding of anti-Gal/GalNAc inhibitable lectin antibodies to wild-type *Neoparamoeba* spp..**

Binding of either monoclonal (3F4) or polyclonal (Shiro) anti-Gal/GalNAc inhibitable lectin antibodies to reduced wild-type *Neoparamoeba* spp. antigen(s) was not detected by Western blot (data not shown). By immunocytochemistry, 3F4 and Shiro bound live, wild-type *Neoparamoeba* spp. (Figs. 5.4 and 5.5), yet these antibodies did not bind to cells that were fixed with SWD. Interestingly, incubation of live trophozoites with galactose or glucose enhanced, rather than inhibited, binding of 3F4 (Fig. 5.4).

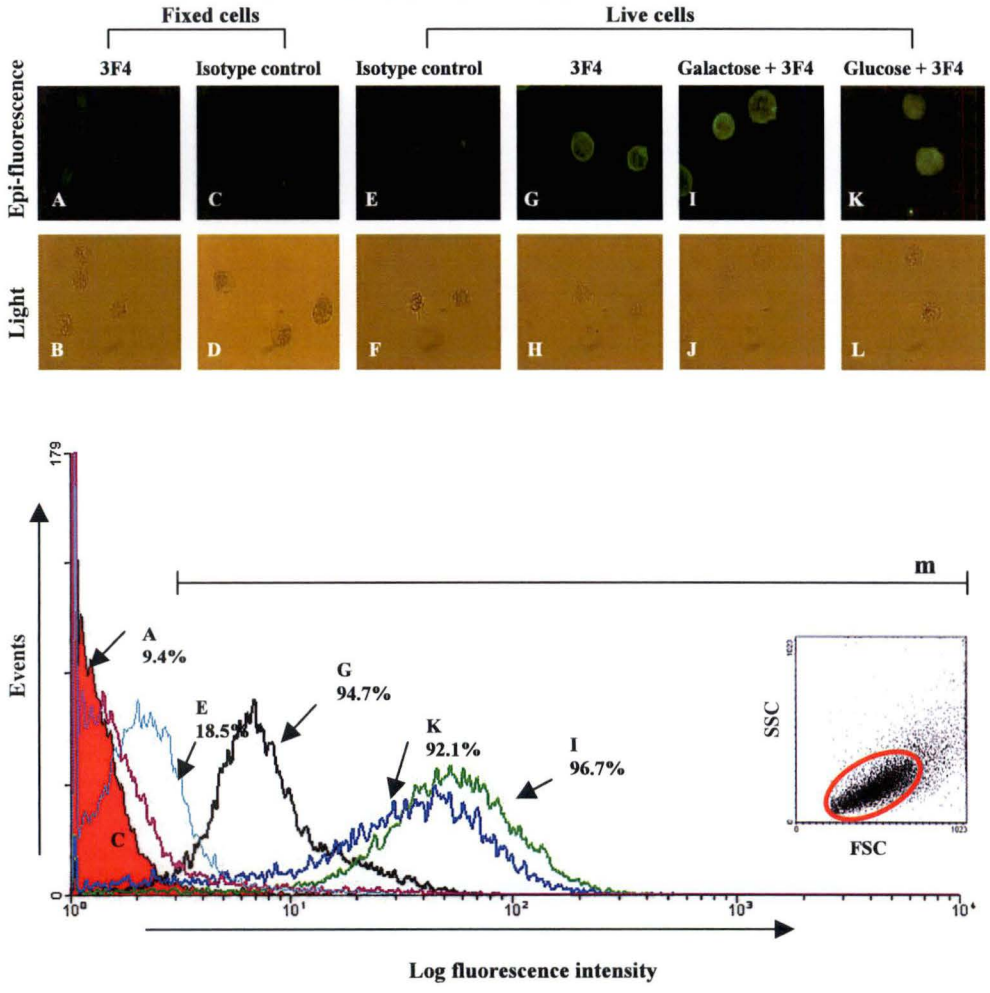


**Figure 5.4**. The monoclonal anti-Gal/GalNAc inhibitable lectin antibody 3F4 bound live (G) but not fixed (A) wild-type *Neoparamoeba* spp. and binding was enhanced by glucose (K) and galactose (I). The light micrographs correspond to the adjacent epi-fluorescent images. A sub-sample from each treatment was photographed before quantitative analysis of the remaining sample by flow cytometry. Therefore the histogram labels correspond to the images above. Analysis of binding of 3F4 to wild-type *Neoparamoeba* spp. by flow cytometry confirmed that 3F4 does not bind fixed cells (A) and fixed cells produce a fluorescence intensity similar to the isotype control of either fixed (C, shaded) or live (E) cells. Binding of 3F4 to live wild-type *Neoparamoeba* spp. produces a fluorescence intensity significantly higher than the isotype control (G) and the intensity increases by approximately 10-fold when cells were incubated in galactose (I) or glucose (K) prior to incubation with 3F4. Bound antibodies were detected with FITC-conjugated goat anti-mouse IgG. Data are representative of cells within the gated region shown in the dot plot (inset). The proportion of cells producing a fluorescence intensity significantly higher than the isotype control of live cells (C) are presented on the histogram and represent data assessed within the marked region (m). Flow cytometric data were analysed and presented using WinMDI 2.8 software.

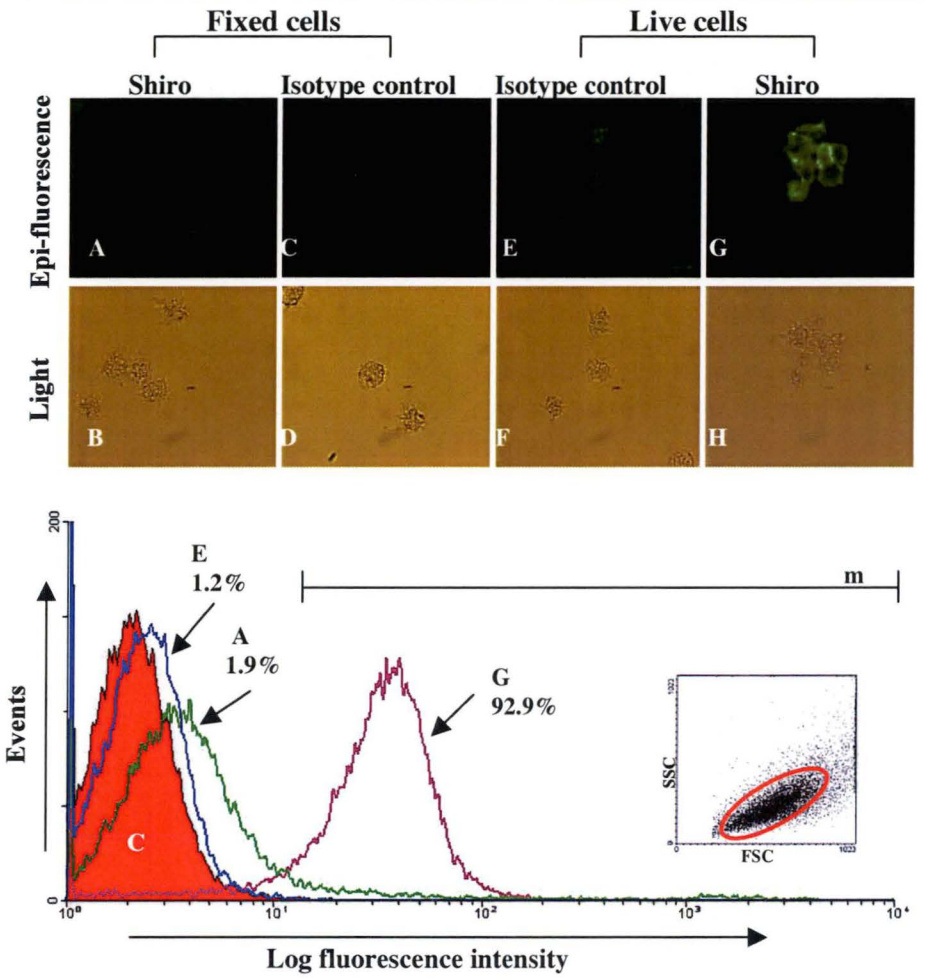


**Figure 5.5.** The polyclonal anti-Gal/GalNAc inhibitable lectin antibody "Shiro" bound live (G) but not fixed (A) wild-type *Neoparamoeba* spp.. The light micrographs correspond to the adjacent epi-fluorescent images. A sub-sample from each treatment was photographed before quantitative analysis of the remaining sample by flow cytometry. Flow cytometry confirmed binding of Shiro to live and not fixed wild-type *Neoparamoeba* spp.. A fluorescence intensity similar to the isotype control of fixed (C, shaded) or live cells (E) is produced by fixed cells probed with Shiro (A). Binding of Shiro to live wild-type *Neoparamoeba* spp. (G) produces a fluorescence intensity significantly higher than the isotype controls. Bound antibodies were detected with FITC-conjugated goat anti-mouse IgG. Data are representative of cells within the gated region shown in the dot plot (inset). The proportions of cells producing a fluorescence intensity significantly higher than the isotype control of live cells (C) are presented on the histogram and represent data assessed within the marked region (m). Flow cytometric data were analysed and presented using WinMDI 2.8 software.

**Discussion**

The ability of wild-type *Neoparamoeba* spp. to elicit AGD after incubation with Atlantic salmon cutaneous mucus was significantly reduced. This suggests that components present in the mucus supernatant may include residues with binding affinity to wild-type *Neoparamoeba* spp. cell-surface constituents. Initial adherence to mucus via the Gal/GalNAc inhibitable lectin in the case of *E. histolytica* provides the first interaction necessary for invasion of the mucosal layer. The interaction of *E. histolytica* with Chinese hamster ovary (CHO) cells (measured by rosette formation) was significantly reduced when amoebae were incubated in crude rat or human colonic mucus (Chadee, et al., 1987). The specificity of the Gal/GalNAc inhibitable lectin of *E. histolytica* for galactose or GalNAc suggests that these sugar residues are present in the colonic mucus.

Saccharides are often effective in blocking amoebae attachment *in vitro*. For example, adherence of *E. histolytica* and *E. dispar* to erythrocytes is significantly reduced in the presence of 55 mM galactose (Boettner, Huston, Sullivan and Petri, 2005). *Hartmannella vermiformis* express an orthologue of the *E. Histolytica* Gal/GalNAc inhibitable lectin (Venkataraman, et al., 1997). The interaction between *H. vermiformis* and *L. pneumophila* is inhibitable by anti-Gal/GalNAc inhibitable lectin antibodies and galactose and GalNAc at concentrations of 10 to 100 mM, identifying that the interaction is likely to be mediated by the Gal/GalNAc lectin (Venkataraman, et al., 1997). In the current study, high concentrations (500 mM) of treatment saccharides were applied to

saturate any cell-surface receptors that bound the saccharides. GalNAc (50 mM), was an exception as the Gal/GalNAc inhibitable lectin of *E. histolytica* has a significantly higher affinity for this saccharide than galactose or galactose containing oligosaccharides (Petri, Haque and Mann, 2002). Increasing saccharide concentration may be associated with a reduction in specificity of the lectin for a particular saccharide. For example, galactose or GalNAc binds specifically to the Gal/GalNAc inhibitable lectin of *E. histolytica*, however agglutination of human erythrocytes induced by *E. histolytica* membrane lectins is inhibited by high concentrations (250 mM) of other saccharides including fucose and mannose (Adler, Wood, Lee, Lee, Petri and Schnaar, 1995). Trapping of live nematodes by the microcarnivorous fungi *Arthrobotrys conoides* is lectin-mediated. Nematode trapping is inhibitable by glucose, mannose, arabinose and maltose at 200 mM yet with an increase in saccharide concentration to 400 mM; GalNAc, GlcNAc, fructose and melbiose also inhibit trapping of nematodes and the authors suggested this may be due to the high saccharide concentration (Rosenzweig and Ackroyd, 1983). To identify if the reduction in AGD pathology observed in the current study was influenced by high concentrations of saccharides, further research is needed and should include applying a lower range of saccharide concentrations than assessed in the current study.

For some parasites, attachment to host tissues may be mediated by a range of lectins or by lectins with multiple binding affinities. For example, agglutination of sheep erythrocytes by the cellular slime mould *Distyostelium discoideum*

lectin (discoidin II) is significantly inhibited by a range of saccharides at 5 mM including D-fucose, D-galactose and GalNAc (Frazier, Rosen, Reitherman and Barondes, 1975). It is possible that high saccharide concentrations may have masked lectin specificity, alternatively wild-type *Neoparamoeba* spp. may employ multiple lectins, or lectins with multiple carbohydrate recognition domains, to colonise Atlantic salmon gill tissues. Further characterisation of ligand(s) that may reduce the ability of wild-type *Neoparamoeba* spp. to colonise Atlantic salmon gill tissues is warranted.

Western blot analyses failed to detect binding of 3F4 or Shiro to reduced wild-type *Neoparamoeba* spp. antigens and this may suggest that antibodies recognise conformational epitopes. Indeed, immunoprecipitation studies indicate that 3F4 binds a conformational epitope of the Gal/GalNAc inhibitable lectin of *E. histolytica* (Pillai, Wan, Yau, Ravdin and Kain, 1999). Western blot was used to confirm the binding of the anti-Gal/GalNAc inhibitable lectin antibody 1G7 to an orthologue of the Gal/GalNAc inhibitable lectin expressed by *H. vermiformis* (Venkataraman, et al., 1997). However the Western blot performed in the current study applied an antigen concentration (cell equivalents) over 100-fold lower than the aforementioned study. Wild-type *Neoparamoeba* spp. are obtained from AGD-affected Atlantic salmon gill tissues. Currently, an AGD co-habitation tank is maintained at the University of Tasmania to supply wild-type *Neoparamoeba* spp. for experimental studies. AGD-naïve Atlantic salmon are regularly introduced to the co-habitation tank and become infected by wild-type

*Neoparamoeba* spp.. Wild-type *Neoparamoeba* spp. are isolated from the gills of Atlantic salmon as they become moribund from AGD, which typically occurs in around 4 weeks. Following the current protocol for isolating gill-derived amoebae (Morrison, et al., 2004), an average of $10^6$ cells may be obtained from 3-4 AGD-affected Atlantic salmon. Applying comparable numbers of wild-type *Neoparamoeba* spp. to that of *H. vermiformis* ($10^7$/lane) for Western blotting would require up to 40 donor fish for Western blot analysis of a single lane, therefore replicating the method of Venkataraman et al., (1997) is impractical. While failure to detect antibody binding may be due to the conformational structure of the epitope(s), it must also be considered that the Western blot conditions applied in the current study may not have been sensitive enough to detect binding.

By immunocytochemistry, both anti-Gal/GalNAc inhibitable lectin antibodies, Shiro and 3F4 failed to bind fixed wild-type *Neoparamoeba* spp.. It has been previously discussed that 3F4 binds a conformational epitope of the Gal/Gal/NAc inhibitable lectin of *E. histolytica*. However, fixation of cells can, in some cases, reduce antibody binding (Van Ewijk, Van Soest, Verkerk and Jongkind, 1984). Atlantic salmon antibodies bind to wild-type *Neoparamoeba* spp. fixed following the same protocol used here (Vincent, Adams, Nowak and Morrison, 2008; Vincent, Nowak and Morrison, 2008) yet the effect of fixation on those target epiotpe(s) is unknown. If binding of 3F4 or Shiro to wild-type

*Neoparamoeba* spp. is dependent on epitope conformation then fixation may influence antibody binding.

Cell-surface molecule(s) of live wild-type *Neoparamoeba* spp. are recognised by the Gal/GalNAc inhibitable lectin antibodies 3F4 and Shiro. Binding of 3F4 to *E. histolytica* is galactose inhibitable (Petri, Jackson, Gathiram, Kress, Saffer, Snodgrass, Chapman, Keren and Mirelman, 1990). In contrast, binding of 3F4 to live wild-type *Neoparamoeba* spp. was enhanced when cells were incubated in galactose or glucose before incubation with primary antibodies. It is unlikely that glucose or galactose stimulate cell-surface receptor expression during the 20 min incubation at 4°C. Glucose and galactose may bind region(s) of cell-surface molecules expressed by wild-type *Neoparamoeba* spp. inducing conformational change(s) that results in an increase of the availability of binding sites for 3F4. Ligand–induced conformational changes of cell-surface receptors used for attachment have been described. For example, antibody binding is enhanced after conformational change of L-selectin induced by the binding of a specific monoclonal antibody (Leid, Steeber, Tedder and Jutila, 2001). In addition, binding of galactose, GalNAc or fucose to mouse macrophage Gal/GalNAc-specific

C-type lectin induces conformational change leading to enhanced antibody binding (Hosoi, Imai and Irimura, 1998). It is possible that the same receptor(s) is/are associated with 3F4 binding and attachment of wild-type *Neoparamoeba* spp. to gill tissues *in vivo*. Ligand-induced conformational change may increase

the availability of binding sites for 3F4 and at the same time reduce the ability of the receptor(s) to bind the target carbohydrate residues present in Atlantic salmon gill tissues. Further investigation is needed to determine the binding site for 3F4 on wild-type *Neoparamoeba* spp..

In conclusion, ligand(s) present in Atlantic salmon mucus may be utilised by wild-type *Neoparamoeba* spp. for colonising the gill tissues. The observed reduction in gill pathology of Atlantic salmon exposed to wild-type *Neoparamoeba* spp. incubated in each of the saccharides assessed appears to be non-specific. While the high saccharide concentrations used here may have masked the specificity of attachment receptor(s), binding of wild-type *Neoparamoeba* spp. to Atlantic salmon gill tissues may be mediated by multiple receptors. Further study is required to elucidate the mechanism(s) associated with the attachment of wild-type *Neoparamoeba* spp. to Atlantic salmon gill tissues.

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# Chapter 6

# General Discussion

Atlantic salmon have been reported to develop resistance to AGD in terms of increased survival and reduced gill pathology (Table 6.1). In isolated cases, resistance of Atlantic salmon to wild-type *Neoparamoeba* spp. under conditions that are favorable for the development of AGD in the laboratory has been observed (fish 1 and fish 2, Chapter 2). However, the predominant factors associated with resistance of Atlantic salmon to AGD in the laboratory include water temperature and prolonged or previous exposure to wild-type *Neoparamoeba* spp.. Previous exposure of Atlantic salmon to wild-type *Neoparamoeba* spp. under conditions where disease is present at a level that does not result in morbidity may enhance the ability of the host to develop resistance. For example, resistance of Atlantic salmon to wild-type *Neoparamoeba* spp. was described in terms of reduced gross gill pathology following secondary exposure to wild-type *Neoparamoeba* spp. at 14°C (Findlay, Helders, Munday and Gurney, 1995; Findlay and Munday, 1998). Furthermore, Atlantic salmon exposed to wild-type *Neoparamoeba* spp. at 12°C for one month demonstrated increased survival to wild-type *Neoparamoeba* spp. challenge at 16°C (Chapter 1).

Initial investigations of the potential of immunostimulants in resistance of Atlantic salmon to AGD showed some promising results. Activation of the innate immune response by immunostimulants may play a role in resistance of Atlantic salmon to AGD in wild-type *Neoparamoeba* spp.. For example, Atlantic salmon that were injected with CpG oligodeoxynucleotides demonstrated >30% higher survival rate than fish injected with PBS (Bridle, Butler and Nowak, 2003). Bath application of

levamisole and in-feed inclusion of β-glucans provided some protection to Atlantic salmon against AGD-associated mortality (Zilberg, Findlay, Girling and Munday, 2000). Further investigations of in-feed inclusion of immunostimulants as a prophylactic treatment for AGD have failed to provide conclusive evidence of their protective qualities. For example, in-feed administration of β-glucans did not enhance AGD-resistance in Atlantic salmon (Bridle, Carter, Morrison and Nowak, 2005). Similarly, in-feed inclusion of other immunostimulants including commercially available EcoActiva and EcoBoost, did not enhance the survival of Atlantic salmon exposed to wild-type *Neoparamoeba* spp. (Nowak, Morrison, Crosbie, Adam, Butler, Bridle, Gross, Vincent, Embar-Gopinath, Carson, Raison, Villavedra, McCarthy, Broady and Wallach, 2004).

While resistance of Atlantic salmon to wild-type *Neoparamoeba* spp. may be observed in terms of enhanced survival, in some cases, the level of gill pathology of groups of putatively resistant fish is similar to groups of fish that do not demonstrate resistance. This suggests that resistance of Atlantic salmon to AGD may be associated with physiological adaptation. It has been observed that 60-70% of gill lamellae of rainbow trout, *Oncorhynchus mykiss* Walbaum, or lingcod, *Ophiodon elongates* Girard, are perfused at rest (Booth, 1979; Farrell, Daxboeck and Randall, 1979). Utilising the remaining 40-70% of gill lamellae may provide a means of increased oxygen uptake by AGD-affected fish therefore off-setting the reduction of gill surface area due to the development of lesions. AGD-affected and AGD-unaffected Atlantic salmon exposed to graded hypoxia demonstrated similar rates of

$O_2$ uptake (Powell, Fisk and Nowak, 2000) suggesting that the reduction of respiratory surface area due to AGD lesions has no adverse effect on respiratory function.

Throughout this project, only two fish (fish 1 and fish 2) were identified to have developed a significant serum antibody response towards wild-type *Neoparamoeba* spp. antigens. Of these two fish, fish one developed serum antibodies that bound peptide antigens while serum antibodies of fish 2 bound carbohydrate antigens of wild-type *Neoparamoeba* spp. (Chapter 2). In the sera of several other AGD-affected Atlantic salmon, low-level antibodies that bound carbohydrate determinants of wild-type *Neoparamoeba* spp. antigens were detected by Western blotting (Chapters 3 and 4).

Interlamellar cysts that develop in AGD-affected Atlantic salmon gill tissues provide an environment for potential interaction of wild-type *Neoparamoeba* spp. and immune cells. Macrophage-like cells have been observed within interlamellar cysts containing *Neoparamoeba* spp. (Adams and Nowak, 2003). Phagocytosis of wild-type *Neoparamoeba* spp. by antigen presenting cells may result in the trafficking of antigens to the lymphoid tissues of the anterior kidney and/or spleen and the subsequent production of a serum antibody response towards antigens of wild-type *Neoparamoeba* spp.. However, given the results obtained throughout this project, antigen processing of wild-type *Neoparamoeba* spp. antigens is either highly limited, or antigen processing and presentation may be affected by immunosuppression.

**Table 6.1.** Comparison of studies where resistance of Atlantic salmon to AGD has been described. The experimental regimes and the proposed factor(s) influencing AGD resistance are summarised.

| | **Findlay et al., 1995** | **Findlay and Munday, 1998** | **Bridle et al., 2003** | **Bridle et al., 2005** | **Chapter 1** | **Chapter 2** |
|---|---|---|---|---|---|---|
| **Salinity (‰)** | NP | NP | 37 | 35 | 35 | 35 |
| **Temperature (°C)** | 14 | 14 | 17 | 16 ± 0.5 | 12 (exposure 1)<br>16 (exposure 2) | 15-16 |
| **Treatment groups** | 1) Naïve<br>2) Exposed 4 w then maintained in FW 4 w | Experiment 1<br>1) Exposed once (4 w) with 2-3 h FW bath<br>2) Naïve<br>Experiment 2<br>1) Exposed twice (4 w) with 2-3 h FW bath between each exposure<br>2) Exposed once (4 w) with 2-3 h FW bath<br>3) Exposed once (4 w) maintained in FW 4 w<br>4) Naïve | Intraperitoneal immunisation<br>1) CpG-ODN<br>2) Non CpG-ODN<br>3) PBS<br>4) Untreated | β-glucan diets<br>1) Diet A<br>2) Diet B<br>3) Diet C<br>4) Control diet | 1) Exposed 4 w, FW bath 24 h<br>2) Naïve | Fish 1<br>Fish 2 |
| **Mode of exposure** | Co-habitation | Co-habitation | Inoculation (2460 cells/L) | Inoculation (1150 cells/L over 3 d) | Inoculation (500 cells/L) | Co-habitation |
| **Duration of challenge (days)** | 28 | 28 | 16 | 72 | 35 | 28-168* |
| **Resistance observed** | Yes – previously exposed fish | Yes – in both experiments previously exposed (once or twice) fish | Yes- CpG-ODN 90% survival | Not treatment specific. Sub population with increased survival and low gill pathology | Previously exposed increased survival. No difference in pathology | Yes- duration of survival, fish 1 low level gill pathology |
| **Proposed factor(s) influencing resistance** | Previous exposure | Previous exposure | Immunostimulation | Inherent resistance, prolonged exposure | Previous exposure | Prolonged exposure |

NP – not provided, FW – fresh water. *estimated duration of exposure

The development of a serum antibody response towards exogenous peptide antigens may occur following a range of antigen presentation scenarios. In addition to the conventional presentation of exogenous peptide antigen(s) by an APC with MHC II, exogenous peptide(s) can be presented by MHC class I, reviewed by Brode and Macary (2004). MHC class I is constitutively expressed by all nucleated cells therefore presentation of wild-type *Neoparamoeba* spp. by MHC class I molecules may provide an alternative means of antigen presentation. In contrast, the development of an antibody response towards exogenous carbohydrate antigens is not necessarily reliant on MHC or T cells as in mice, a serum antibody response to a carbohydrate antigen can develop independently of T cells and MHC. T cell-independent (TI) antigens consist of two types, TI-1 and TI-2. TI-1 antigens are mostly mitogens such as lipopolysaccharide (LPS), while TI-2 antigens are not mitogens and include highly repetitious polymeric molecules. TI antigens induce B cell activation yet neither TI-1 or TI-2 antigens induce immunological memory (Goldsby, Kindt, Osborne and Kuby, 2003) and therefore an antibody response towards TI antigens would not be enhanced following secondary exposure to the TI antigen.

As an example of a T-cell independent antibody response to a carbohydrate antigen, T-cell deficient mice (athymic nude, TCRβ -/-, MHC II -/-, and CD40-/-) i.p injected with a carbohydrate antigen of the tapeworm, *Echinococcus multilocularis*, developed a specific antibody response to the target antigen (Dai,

Hemphihll, Waldvogel, Ingold, Deplazes, Mossman and Gottstein, 2001). The majority of carbohydrate antigens are considered TI antigens (Cobb and Kasper, 2005). Carbohydrate residues are immunodominant cell-surface molecules of wild-type *Neoparamoeba* spp. in mice (Villavedra, Lemke, To, Broady, Wallach and Raison, 2007) and numerous AGD-affected Atlantic salmon possessed serum antibodies that bound carbohydrate antigens of wild-type *Neoparamoeba* spp.. However, there was a single case where an Atlantic salmon (fish 2) developed high-titre antibody response to carbohydrate antigenic determinants of wild-type *Neoparamoeba* spp. (Chapter 2). As TI antigens do not induce immunological memory, the development of a high-titre antibody response of fish 2 may have been mediated via recognition of carbohydrate residue(s) of a glycoprotein(s) presented by MHC II.

The fish identified in studies throughout this project that developed a serum antibody response towards wild-type *Neoparamoeba* spp., had been exposed to wild-type *Neoparamoeba* spp. for >4 months (Table 6.2) and very few fish with detectable serum antibodies were identified. A serum antibody response in fish following an ectoparasitic infestation may be slow to develop. For example, a serum antibody response towards ectoparasitic mussel larvae in large mouth bass, *Micropterus salmonides* Lacepède, exposed twice to *Lampsilis reeveiana* Simpson, peaked 60 days post-initial exposure (Grayson, et al., 1991). Antibodies that bind the monogenean, *Discocotyle sagittata* Leuckart, were detected in the sera of rainbow trout, exposed to *D. sagittata* during pond culture

with heightened antibody responses observed in some fish after >1 year (Rubio-Godoy, et al., 2003a). Similarly, the development of a serum antibody response in sea-farmed Atlantic salmon after multiple infestations of sea lice, *Caligus elongates* Nordmann, may take up to 2 years (Grayson, et al., 1991). Similar results were observed in AGD-affected Atlantic salmon with detectable serum anti-*Neoparamoeba* spp. antibodies detected in some fish after 280 days of sea cage culture (Chapter 3). It must also be considered that the fact that few fish have been identified to develop a serum anti-*Neoparamoeba* spp. antibody response may be a result of the low-level exposure of wild-type *Neoparamoeba* spp. antigens to the immune system (occurring infrequently through the internalisation of parasites), or be due a reduction of antigen processing and presentation activity due to immunosuppression. Immunosuppression of AGD-affected Atlantic salmon due to stress is unlikely. For example assessment of the stress response in AGD-affected Atlantic salmon provided no evidence that Atlantic salmon with AGD are stressed, as plasma cortisol levels were within the range of non-stressed fish (Fazioli, 2005). Down-regulation of genes involved in antigen processing and presentation is observed in gill lesions of AGD-affected Atlantic salmon (Young, et al., 2008a) and this may be responsible for the overall lack of the development of acquired immune response in AGD-affected Atlantic salmon.

Serum anti-*Neoparamoeba* spp. antibodies with measurable activity by an ELISA were detected in Atlantic salmon exposed to wild-type *Neoparamoeba*

spp. by co-habitation in excess of 4 months (Table 6.2). These cases are unique as fish exposed to wild-type *Neoparamoeba* spp. in the laboratory generally succumb to AGD within 4 weeks (Bridle, et al., 2003; Gross, Morrison, Butler and Nowak, 2004; Bridle, et al., 2005; Morrison and Nowak, 2005). The induction of AGD in Atlantic salmon in the laboratory is relatively rapid. Atlantic salmon are exposed to high cell densities of wild-type *Neoparamoeba* spp. under conditions that are favorable for development of AGD. Furthermore, the use of recirculation systems augments the perpetual infection through horizontal transfer of amoebae, contributing to the level of wild-type *Neoparamoeba* spp. infection. Atlantic salmon exposed to wild-type *Neoparamoeba* spp. in the laboratory experience a short infection time before morbidity and this may be linked to the lack of antibody response that is predominately observed. In contrast, in the marine culture environment, parasite numbers and environmental parameters are dynamic. Sea-cage cultured Atlantic salmon are predominately exposed to wild-type *Neoparamoeba* spp. during summer months. During these times, wild-type *Neoparamoeba* spp. infection is maintained at a low level by fresh water bathing. While Atlantic salmon are exposed to wild-type *Neoparamoeba* spp. many times during the marine culture period only low-level anti-*Neoparamoeba* spp. antibodies were detected in the serum of some fish after 13 months of sea-cage culture (Table 6.2). The absence of significant anti-*Neoparamoeba* spp. antibody response in marine-cultured Atlantic salmon may be attributed to the infection being maintained at a low level.

**Table 6.2.** Atlantic salmon exposed to wild-type *Neoparamoeba* spp. in excess of 72 days may develop serum anti-*Neoparamoeba* spp. antibodies. The mode and duration of exposure, number of fish sampled and the proportion of sero-positive samples are summarised.

| | Mode of exposure | Duration of exposure (days) | Number fish assessed | Number sero-positive** |
|---|---|---|---|---|
| **Chapter 1** | | | | |
| Exposure 1 | L | 28 | 30 | 0 |
| Exposure 2 | L | 35 (total 63) | 22 | 11 |
| **Chapter 2** | | | | |
| UTAS infection tank | L | 28-168^ | 17 | 2[1] |
| Bridle et al., 2006 | L | 72 | 63 | 3 |
| AGD infection | L | 23 | 23 | 0 |
| **Chapter 3** | | | | |
| Cultured stock* | F | 224 | 40 | 5 |
| Cultured stock* | F | 280 | 40 | 25 |
| Cultured stock* | F | 364 | 40 | 23 |
| Broodstock | F | 420 | 55 | 45 |
| **Overall total** | | | **330** | **114** |

** sero-positive by Western blot, * combined data of triploid and diploid cultured Atlantic salmon. L – laboratory, F – field, ^estimated exposure, [1] antibody activity measurable in an ELISA in fish exposed >90 days.

A heightened antibody response towards wild-type *Neoparamoeba* spp. may be observed if sea-cage cultured fish were monitored over a longer period of time. Extending the duration of exposure of fish to an ectoparasite may provide further opportunity for the development of an antibody response. Inoculating fish holding systems maintained at 12°C with wild-type *Neoparamoeba* spp. was successful in eliciting AGD in AGD-naïve Atlantic salmon with no fish mortality over a period of 28 days, while at 16°C morbidity of AGD-naïve Atlantic salmon can exceed 50% after 35 days (Chapter 2). Further research to assess the development of an antibody response in AGD-affected Atlantic salmon may

require fish to be exposed to wild-type *Neoparamoeba* spp. in excess of 12 months under sub-lethal conditions, perhaps at a water temperature below 12°C.

Fish gills may be considered immune-reactive, for example, immersion vaccination with bacterial antigens stimulates a gill-associated antibody response. Antibody-secreting cells were observed in abundence in the gills of sea bass following immersion vaccination (dos Santos, Taverne-Thiele, Barnes, van Muiswinkel, Ellis and Rombout, 2001). In addition, higher levels of parasite-specific antibodies are found in gill tissues compared to antibody levels detected in the peripheral blood of rainbow trout following secondary immersion vaccination with *Flavobacterium branchiophilum* (see Lumsden, Ostland, MacPhee and Ferguson, 1995).

While immersion of Atlantic salmon in a low concentration of wild-type *Neoparamoeba* spp. antigen, failed to provide protection against AGD (Morrison et al., 2005), uptake of antigens via the gills was not assessed. Antigens of wild-type *Neoparamoeba* spp. may not be readily taken up by through the gills of Atlantic salmon. Assessment of the uptake of wild-type *Neoparamoeba* spp. antigen via the gills following immersion vaccination may provide further insight useful for AGD vaccine research.

In the case of AGD-affected Atlantic salmon, immune-like cells are present within lesions (Adams and Nowak, 2004) although very few $Ig^+$ cells have been

observed (Gross, 2006). Cells expressing MHC class II are present in the gill tissues of AGD-affected Atlantic salmon (Morrison, Koppang, Hordvik and Nowak, 2006), however it appears unlikely that a localised response would develop at the site of infection as there is a significant down-regulation of antigen processing machinery in the gills of AGD-affected Atlantic salmon (Young et al., 2008).

Despite the overall lack of antibody response towards wild-type *Neoparamoeba* spp. in AGD-affected Atlantic salmon, immunisation with wild-type *Neoparamoeba* spp. antigens may elicit a significant antibody response. For example, a modest increase in antibody titre was observed in Atlantic salmon immunised with an increase in concentration of *N. pemaquidensis* antigen (Bryant et al., 1995). In some cases, some level of resistance has been observed in fish immunised with parasite antigen(s). Rainbow trout immunised with antigens of the *D. sagittata* conferred resistance to parasitism to over 50% of immunised fish (Rubio-Godoy, Sigh, Buchmann and Tinsley, 2003b). Furthermore, antibody-mediated protection against white spot disease in channel catfish caused by *I. multifiliis* and grouper caused by *Cryptocaryon irritans* Brown, is associated with parasite-specific antibodies in the skin of host fish (Xu and Klesius, 2002; Xu and Klesius, 2003; Yambot and Song, 2006; Luo, Xie, Zhu and Li, 2007).

While at this stage we have observed only two isolated cases of Atlantic salmon with high-titre serum anti-*Neoparamoeba* spp antibodies and low-level gill pathology. Immunisation of Atlantic salmon with the appropriate concentration of *N. perurans* antigen(s) may elicit a high-titre antibody response. This may lead to resistance in terms of reduced mortality and reduced gill pathology.

Immunisation of fish by a range of routes can result in the presence of mucosal antibodies. For example, immersion of channel catfish in a bath containing dinitrophenylated-horse serum albumin ($DNP_{16}$-HoSA) elicits a mucosal antibody response towards $DNP_{16}$-HoSA (Lobb, 1987) and bath immunisation of rainbow trout with *F. branchiophilum* stimulated a significant gill-associated antibody response (Lumsden, et al., 1995). Further, oral or bath immunisation of ayu, Plecoglossus altivelis Temminck and Schlegel, with V. anguillarum stimulated a significant mucosal antibody response that inhibited adhesion of V. anguillarum to ayu skin in vitro (Kawai, Kusuda and Itami, 1981). Oral immunisation of spotted sand bass Paralabrax maculatofasciatus Steindachner, with an extracellular lectin of Aeromonas veronii stimulated a secretory antibody response in skin mucus, intestinal mucus and bile (Merino-Contreras, Guzman-Murillo, Ruiz-Bustos, Romero, Cadena-Roa and Ascencio, 2001). Intraperitoneal (i.p) immunisation can also stimulate the development of a mucosal antibody response (LaFrentz, LaPatra, Jones, Congleton, Sun and Cain, 2002).

At times, mucosal antibodies can be detected before serum antibodies despite the route of antigen delivery. For example, in the European eel, *Anguilla anguilla* L., a peak antibody response was detected in mucus before sera following immersion vaccination with Vulnivaccine (Esteve-Gassent, Nielsen and Amaro, 2003). In addition, rainbow trout immunised i.p with FITC-keyhole limpet haemocyanin (KLH), a peak antibody response was detected in mucus before serum (Cain, Jones and Raison, 2000). Identified in studies with fugu, transport of IgM from the skin to the mucus occurs via a polymeric Ig receptor (pIgR) (Hamuro, Suetake, Saha, Kikuchi and Suzuki, 2007). Therefore, the induction of a serum antibody response may also result in the presence of mucosal antibodies via transfer of IgM by pIgR.

While limited attempts to detect a mucosal antibody response in AGD-affected Atlantic salmon have been made (Findlay, et al., 1995; Vincent, Morrison and Nowak, 2006), all have failed. If transport of IgM to the mucus via a pIgR occurs in Atlantic salmon, then failure to detect mucosal antibodies may be associated with the presence of low levels in the sera leading to low or no transfer of IgM to the mucus. It must also be considered that the methods used to detect antibodies in serum of AGD-affected Atlantic salmon may not be effective in detecting low-level antibodies in mucus. Mucosal antibodies may be detectable in Atlantic salmon after the development of a high-titre anti-*Neoparamoeba* spp. antibody response. However, the presence of antibodies in the mucus of the 2 fish that developed a high-titre antibody response towards wild-type *Neoparamoeba* spp. was not assessed.

While carbohydrate antigens of wild-type *Neoparamoeba* spp. appear immunodominant in AGD-affected Atlantic salmon, an antibody response towards peptide antigens of wild-type *Neoparamoeba* spp. may be elicited in the sera and/or mucus of Atlantic salmon by immunisation. While the activity of such antibodies at the site of infection remains questionable, antibodies have been shown to maintain the ability to bind to target epitope(s) in sea water. For example, monoclonal antibodies that bind the vitelline layer of the sea urchin, *Strongylocentrotus purpuratus* Stimpson, egg inhibit fertilization by blocking binding of sperm to the egg surface although only a small proportion of these monoclonal antibodies were able to bind in sea water (Gache, Niman and Vacquier, 1983). Serum and mucosal antibodies of barramundi, *Lates calcarifer* Bloch, immunised with *Streptococcus iniae*, bind to antigens of *S. iniae* in salinities similar to sea water (Delamare-Deboutteville, Wood and Barnes, 2006). In contrast, results presented by Bricknell, Bisset and Bowden (2002) suggested that optimal binding of Atlantic salmon antibodies was within the range of pH 7 to 8 and at an osmolality similar to physiological, between 100 and 400 $mOsml^{-1}$. MBP is a useful ligand for isolating serum IgM from teleosts including tomato clown fish (Cobb, Levy and Noga, 1998) barramundi, (Crosbie and Nowak, 2002) and rockfish, *Sebastes schlegeli* Higendorf, (Shin, Lee and Palaksha, 2006). Bricknell et al., (2002) did not assess if the binding of Atlantic salmon IgM to MBP was mediated by the Fc or the Fab regions, therefore the level of antibody binding detected may not be representative of Fab binding. Therefore

the ability of Atlantic salmon antibodies to bind target epitope(s) in seawater can not be inferred from the work of Bricknell et al., (2002).

Preliminary assessment of the fate of a monoclonal antibody (3F4) that bound wild-type *Neoparamoeba* spp. suggested that antibodies do not remain attached to live wild-type *Neoparamoeba* spp. in sea water (Appendix 3). The antibodies may have been shed, pinocytosed and destroyed or rejected, or simply antibodies lost the ability to bind the cell-surface of wild-type *Neoparamoeba* spp. in sea water due to conformational change(s). The binding of salmon anti-*Neoparamoeba* spp. antibodies was also assessed and while antibody binding was observed after wild-type *Neoparamoeba* spp. were suspended in sea water for 1 h, consistent results were not attained in repeated assays (data not shown). The effect of antibodies on wild-type *Neoparamoeba* spp. *in vivo* may be influenced by antibody titre and/or the specificity of the antigenic determinants therefore further research in this area is warranted. Such assays should assess the binding of Atlantic salmon anti-*Neoparamoeba* spp. antibodies to wild-type *Neoparamoeba* spp. over time, across a range of salinities.

As previously discussed (Chapter 1), vaccination studies with crude wild-type *Neoparamoeba* spp. preparations are impractical, therefore other approaches are needed for the identification of candidate vaccine antigens for an anti-AGD vaccine. Taking the approach of screening host sera to identify candidate vaccine antigens for an anti-AGD vaccine was less effective than anticipated. Serum

antibodies from a single AGD-affected Atlantic salmon (fish 1) bound peptide antigenic determinants of wild-type *Neoparamoeba* spp.. It may be possible to identify these antigenic determinants by cDNA expression library screening and this is currently being pursued in our laboratory. If antibodies present in the serum of fish 1 detect recombinant wild-type *Neoparamoeba* spp. peptide(s), further assessment of the recombinant protein(s) as candidate anti-AGD vaccine antigen(s) is warranted. Laboratory testing of DNA vaccines for AGD have shown modest protection of Atlantic salmon against wild-type *Neoparamoeba* spp. (Cook, Campbell, Patil, Elliott and Prideaux, 2007). However whether protection is mediated via the induction of an adaptive immune response is unknown. The role of adaptive immunity in resistance of Atlantic salmon to AGD is central for the development of an anti-AGD vaccine and measurement of adaptive immune parameters including serum and mucosal antibodies should be conducted in parallel with challenge studies.

Colonisation of wild-type *Neoparamoeba* spp. on Atlantic salmon gill tissues may occur passively or be mediated via specific ligand interactions. To explore the presence of lectin-mediated colonisation of wild-type *Neoparamoeba* spp. to Atlantic salmon gill tissue, trophozoites were incubated in mucus or a range of saccharides before inoculating fish holding systems containing AGD-naïve Atlantic salmon (Chapter 5). Interestingly a significant reduction in AGD pathology compared to the control was seen with all treatments. High concentrations of saccharides, as used in Chapter 5, may produce a non-specific

inhibitory effect. Therefore assessment of low concentrations of each saccharide is warranted to identify if the inhibition observed may be specific.

Antibodies that bind the *E. histolytica* Gal/GalNAc inhibitable lectin bound the cell-surface of wild-type *Neoparamoeba* spp.. Given this, it was hypothesised that wild-type *Neoparamoeba* spp. may employ a cell-surface lectin to attach to gill tissue. However an orthologue of the *E. histolytica* Gal/GalNAc inhibitable lectin expressed by wild-type *Neoparamoeba* spp. was not identified by homology cloning (Appendix 2). A number of monoclonal antibodies that bind the Gal/GalNAc inhibitable lectin of *E. histolytica* have been described and include antibodies that bind linear or conformational epitopes. The monoclonal antibody assessed in Chapter 5 (3F4) binds a conformational region of the Gal/GalNAc inhibitable lectin (Mann, Chung, Dodson, Ashley, Braga and Snodgrass, 1993). Assessment of the ability of MAbs that bind linear epitope(s) of the *E. histolytica* Gal/GalNAc inhibitable lectin to bind live wild-type *Neoparamoeba* spp. may identify orthologous cell-surface molecules useful for vaccine candidate antigens. Another approach towards detection of an orthologue of the Gal/GalNAc inhibitable lectin expressed by wild-type *Neoparamoeba* spp. may include screening of a wild-type *Neoparamoeba* spp. cDNA library with Gal/GalNAc lectin probes or polyclonal and/or monoclonal Gal/GalNAc antibodies.

It is possible that initial colonisation of wild-type *Neoparamoeba* spp. on the gill tissues of Atlantic salmon is passive (entrapment in mucus) and the subsequent development of pathology may be reliant on ligand-mediated interactions. If the development of AGD pathology is ligand-mediated, these ligand(s) would be common to all fish susceptible to AGD including marine-farmed turbot, *Psetta maxima* L., (Dyková, Figueras and Novoa, 1995; Dyková, Figueras, Novoa and Casal, 1998) European sea bass, *Dicentrarchus labrax* L., and Blue warehou, *Seriolella brama* Gűnther, (Adams, Villavedra and Nowak, 2008). Molecules associated with colonisation of wild-type *Neoparamoeba* spp. *in vivo* may represent novel candidate vaccine antigens for an anti-AGD vaccine. However, induction of a vaccine-induced antibody response may not be protective unless high-titre antibodies are present at the host-parasite interface.

The presence of serum anti-*Neoparamoeba* spp. antibodies is not associated with AGD-resistance of Atlantic salmon. The fact that a high-titre serum anti-*Neoparamoeba* spp. antibodies were detected in just two fish suggests that either processing and presentation of wild-type *Neoparamoeba* spp. antigens was successful in these individual fish or that these antibodies are natural or cross-reactive antibodies that bind to wild-type *Neoparamoeba* spp. antigen(s). As the antigen processing and presentation machinery is significantly down-regulated in the gills of AGD-affected Atlantic salmon, it is questionable whether a serum antibody response could develop. However, the down-regulation of antigen processing machinery may be localised and away from the gill tissues, antigen

processing cells (APCs) may regain normal function. Further elucidation of the development of a systemic antibody response could include tracking wild-type *Neoparamoeba* spp. antigen uptake to identify if antigen(s) reach the lymphoid tissues of the anterior kidney or spleen.

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# Appendix 2

# Assessment of the expression of a Gal/GalNAc inhibitable lectin orthologue by wild-type *Neoparamoeba* spp.

Lectin-mediated attachment may be employed by parasites for attachment to the host. For example, attachment of the human pathogenic amoebae *Entamoeba histolytica* to colonic mucins is mediated by the Gal/GalNAc inhibitable lectin (Ravdin and Guerrant, 1981; Ravdin, John, Johnston, Innes and Guerrant, 1985; Chadee, Petri, Innes and Ravdin, 1987). The Gal/GalNAc inhibitable lectin has shown potential as a vaccine antigen against amebiasis in animal models (Zhang, Cieslak and Stanley, 1994; Soong, Kain, Abd-Alla, Jackson and Ravdin, 1995; Dodson, Lenkowski, Eubanks, Jackson, Napodano, Lyerly, Lockhart, Mann and Petri, 1999). Similarly, *Hartmannella vermiformis* express an orthologue of the Gal/GalNAc inhibitable lectin (Venkataraman, Haack, Bondada and Kwaik, 1997). *H. vermiformis* are ancestrally related to *N. pemaquidensis* and *N. aestuarina* (see Peglar, Amaral Zettler, Anderson, Nerad, Gillevet, Mullen, Frasca, Silberman, O'Kelly and Sogin, 2003). We therefore hypothesised that wild-type *Neoparamoeba* spp. may express an orthologue of the Gal/GalNAc inhibitable lectin. However, an orthologue of the Gal/GalNAc expressed by wild-type *Neoparamoeba* spp. was not identified here by homology cloning.

Wild-type *Neoparamoeba* spp. were isolated from AGD-affected Atlantic salmon as previously described (Morrison, Crosbie and Nowak, 2004). Amoebae were placed in Trizol (Invitrogen) and RNA was extracted following the manufacturer's instructions. The RNA quality and concentration was assessed by agarose gel electrophoresis and spectrophotometeric analysis. Wild-type *Neoparamoeba* spp. cDNA was produced using 650 μg total RNA and Superscript III reverse transcriptase (Invitrogen) following the manufacturer's instructions.

Actin was used as a positive control for the reverse transcription reaction. Wild-type *Neoparamoeba* spp. actin was amplified using the sense (ActN2) and anti-sense (1354R) primers described by Fahrni et al., (2003). The PCR conditions were as follows: 1 cycle of 94°C for 3 min, 35 cycles of 94°C for 30 s, gradient 48-58°C for 30 s and 72°C for 50 s, followed by 5 min at 72°C. PCR products were gel-purified (Qiagen). PCR products were sequenced using either the corresponding forward or reverse primers, the Applied Biosystems BigDye Terminator Ready Reaction kit (version 3.1) and reactions were analysed in the Applied Biosystems 3730×1 DNA analyser (Applied Biosystems, Scoresby, Victoria, Australia).

To assess the expression of an orthologue of the Gal/GalNAc inhibitable lectin by wild-type *Neoparamoeba* spp., primer pairs were designed from a sequence alignment of the *E. histolytica* (GenBank L14815) and *E. dispar* Gal/GalNAc inhibitable lectin (GenBank U73710). Conserved regions of the carbohydrate

recognition domain (Pillai, Wan, Yau, Ravdin and Kain, 1999) were targeted for primer design. As an orthologue of the *E. histolytica* Gal/GalNAc inhibitable binding lectin was isolated from *H. vermiformis* using a monoclonal anti-Gal/GalNAc inhibitable antibody, the region that this antibody (1G7) binds (Mann, Chung, Dodson, Ashley, Braga and Snodgrass, 1993) was also targeted and a set of primers were designed within this region (Fig. A2.1., Table A2.1.). Gradient PCRs were performed for each primer pair with a range of annealing temperatures between 45-60°C. PCR products were assessed by agarose gel electrophoresis. Where the PCR produced an amplicon of the expected size, the products were gel-purified and either directly sequenced or ligated into pGEM-T easy plasmid vector (Promega, Annadale, Australia). After transformation into *E. coli* (DH10β), positive clones were selected for ampicillin resistance. Clones were inoculated into LB broth and plasmids were purified (QIAprep Spin miniprep kit, Qiagen). M13 Forward or M13 reverse oligonucleotides were used for sequencing reactions of plasmid DNA. Sequences were analysed as described above.

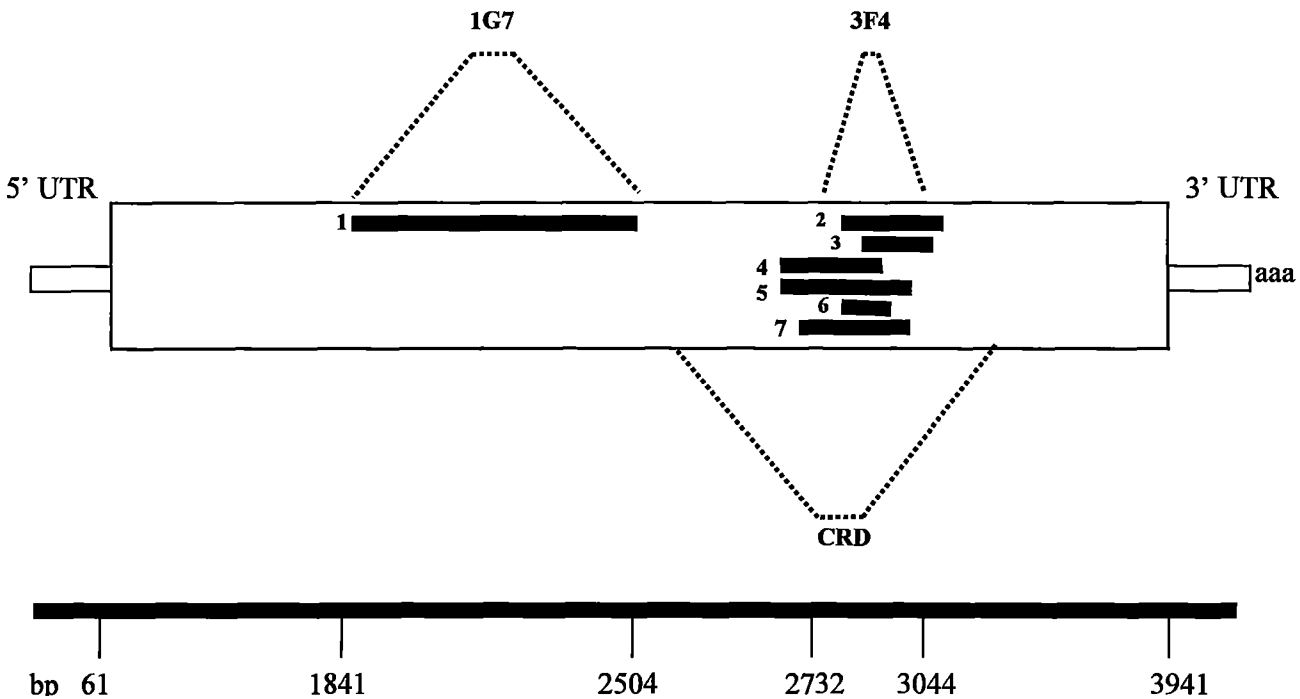


**Figure A2.1.** Diagrammatic representation of the location of primer pairs designed against a sequence alignment of the *E. histolytica* and *E. dispar* Gal/GalNAc inhibitable lectin sequence. Regions are represented by base-pair location of the mRNA. Regions with epitope(s) for the monoclonal antibodies (MAb) 1G7 and 3F4 (Mann, et al., 1993) and the carbohydrate recognition domain (CRD) of the Gal/GalNAc inhibitable lectin (Pillai, et al., 1999) are indicated.

**Table A2.1.** Primers designed for the PCR amplification of a Gal/GalNAc inhibitable orthologue of wild-type *Neoparamoeba* spp.. Target regions 1-7 (Figure A2.1) and their expected amplicon size (base pairs), sense and antisense primers were designed from conserved regions of a sequence alignment of the *E. histolytica* and *E. dispar* Gal/GalNAc inhibitable lectin sequence as shown in Figure A2.1.

| Target region | Expected amplicon size (bp) | Sense (5'-3') | Antisense (5'-3') |
|---|---|---|---|
| 1 | 1198 | ATACACAGCAGGAACAGGAC | GAACAATCAACATTCTCCCAA |
| 2 | 830 | ATGAAAGAGGCAGACAAAGAAG | AGAACTGATGAACCGTCAAGAT |
| 3 | 795 | GAATTTATGAAAGAGGCAGAC | ACATCTTCCTTGATTTGGTG |
| 4 | 320 | GTTCAACAAACACCAGCAT | GCCTCTTTCATAAATTCTTCAC |
| 5 | 763 | GTTCAACAAACACCAGCAT | CTTTACATCCATAATCTACTTCT |
| 6 | 199 | GAATTTATGAAAGAGGCAGAC | GGTTGACAATCAAAGTTAGG |
| 7 | 557 | GTTCAACAAACACCAGCAT | TGTTTTTATTCCATCTTTTTCT |

Using the primer pair ActN2/1354R, a PCR product of around 800 bp was amplified. Sequence data were assessed for identity by BLASTn search and the sequence (830 bp) shared significant sequence identity with eukaryotic actin genes. The sequence has been submitted to GenBank (EU089662). Primer pairs for amplicons 3, 4, 6 and 7 produced PCR products similar to the expected amplicon size. BLASTn and pair-wise comparisons with the *E. histolytica* Gal/GalNAc inhibitable lectin sequence were performed. No significant orthology with the Gal/GalNAc inhibitable lectin of *E. histolytica* was identified.

Under the conditions applied in the current study, expression of an orthologue of the Gal/GalNAc inhibitable lectin of *E. histolytica* by wild-type *Neoparamoeba* spp. was not identified. An orthologue of the *E. histolytica* Gal/GalNAc inhibitable lectin was described for *H. vermiformis* using the monoclonal antibody 1G7 that binds the Gal/GalNAc inhibitable lectin of *E. histolytica* in the region of 1841-2504 (bp) that is outside the carbohydrate recognition domain (CRD) (Pillai, et al., 1999). Primer pair 1 used in the current study was positioned within this region while primer pairs 2-7 were positioned within the CRD and the region that codes for the Gal/GalNAc inhibitable lectin epitope 1 of *E. histolytica* that monoclonal antibody 3F4 binds (Mann, et al., 1993). *E. histolytica* is ancestrally related to *H. vermiformis* (Fahrni, et al., 2003; Peglar, et al., 2003) and binding of 1G7 to an orthologous lectin of *H. vermiformis* suggests that the Gal/GalNAc inhibitable lectin is conserved between these

species. *N. pemaquidensis* and *N. aestuarina* are ancestrally related to *H. vermiformis* (see Peglar, et al., 2003). The failure to amplify an orthologue of the Gal/GalNAc inhibitable binding lectin of *E. histolytica* may be attributed to primer design. Alternatively, an orthologue of the Gal/GalNAc inhibitable lectin may not be expressed by *Neoparamoeba* spp., or in the process of evolution, expression may have been lost.

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# Appendix 3

# Binding of anti-*Neoparamoeba* spp. antibodies to wild-type *Neoparamoeba* spp. in sea water

Attachment of a range of parasites to host cells is mediated by the binding of parasite lectins to carbohydrate residues of host tissues. In many instances, these interactions can be inhibited by blocking the carbohydrate recognition domain (CRD) of the lectin antibodies that bind the CRD. Attachment of the human pathogenic amoebae *Entamoeba histolytica* to colonic mucins is mediated by the Gal/GalNAc inhibitable lectin (Ravdin and Guerrant, 1981; Ravdin, John, Johnston, Innes and Guerrant, 1985; Chadee, Petri, Innes and Ravdin, 1987). Monoclonal antibodies that bind the *E. histolytica* Gal/GalNAc inhibitable lectin block lectin activity of *E. histolytica in vitro* (see Ravdin, Petri, Murphy and Smith, 1986; Venkataraman, Haack, Bondada and Kwaik, 1997; Kwaik, Venkataraman, Harb and Gao, 1998). Monoclonal anti-Gal/GalNAc inhibitable lectin antibodies (3F4) bind the cell surface of wild-type *Neoparamoeba* spp. (Chapter 5). The question raised here is could antibodies that bind the cell-surface of wild-type *Neoparamoeba* spp. *in vitro* remain bound bind in seawater?

Wild-type *Neoparamoeba* spp. were isolated from AGD-affected Atlantic salmon following the method outlined by Morrison et al., (2004) and for the following experiments, both live and fixed wild-type *Neoparamoeba* spp. were used. Live wild-type *Neoparamoeba* spp. were washed 1 × in PBS and incubated with

monoclonal anti-Gal/GalNAc inhibitable lectin antibody 3F4 or monoclonal IgG isotype control (Sigma) at 100 μg/mL for 20 min at 4°C. Antibodies were diluted in 0.1% BSA-PBS Cells were then washed 1 × in PBS and either fixed in SWD (500 μL) or transferred to 0.2 μm-filtered sea water (5 mL) and maintained in suspension by gentle rocking for 1 h at 20°C. Cells that were fixed immediately after incubation with primary antibodies were washed 3 × in PBS. Cells that were transferred to sea water were concentrated by centrifugation for 5 min at 500× *g* and washed 1 × in PBS. Sea water-incubated cells were then fixed and washed 3 × in PBS. Bound antibodies were detected with FITC-conjugated goat anti-mouse IgG (Sigma) following the manufacturer's instructions. A sub-sample of cells from each treatment was photographed (Leica DC300F, Leica Microsystems, Wetzlar, Germany) using light and epi-fluorescence microscopy. The proportion of wild-type *Neoparamoeba* spp. expressing epitope(s) to which the anti-Gal/GalNAc inhibitable lectin antibodies bound was quantified using flow cytometry (Coulter Epics, Beckman Coulter, USA). Ten thousand cells were assessed per treatment and data were analysed using WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA).

Binding of the monoclonal anti-*E. histolytica* Gal/GalNAc antibody, 3F4, to the cell-surface of wild-type *Neoparamoeba* spp. was observed immediately following incubation. However 3F4 was not detected on the cell-surface of wild-type *Neoparamoeba* spp. after cells were suspended in sea water for one hour (Fig. A3.1). The binding of 3F4 to wild-type *Neoparamoeba* spp. may be disrupted in sea water.

Alternatively, wild-type *Neoparamoeba* spp. may have internalised bound antibodies and subsequently the antibodies were degraded or released into the water over the one-hour period. While capping of surface bound antibodies by *Neoparamoeba* spp. was not observed in the current study, *E. histolytica* rapidly redistributes surface bound antibodies, forming caps (Calderon, de Lourdes Munoz and Acosta, 1980). Disruption of protein synthesis in *E. histolytica* for 2 h by cycloheximide completely abolished binding of mucins (Chadee, Johnson, Orozco, Petri and Ravdin, 1988) suggesting that the turn over of cell-surface receptors of *E. histolytica* is rapid. *Neoparamoeba* spp. may also rapidly replenish cell membrane constituents to which 3F4 antibodies bind. Despite the factors associated with the loss of antibody on the cell-surface of wild-type *Neoparamoeba* spp., this may have implications for the potential of antibody-mediated protection of Atlantic salmon against wild-type *Neoparamoeba* spp.. However these data represent a single observation and to elucidate the potential effect(s) of salmon antibodies on the ability of wild-type *Neoparamoeba* spp. to colonise Atlantic salmon gill tissues, further research is warranted.

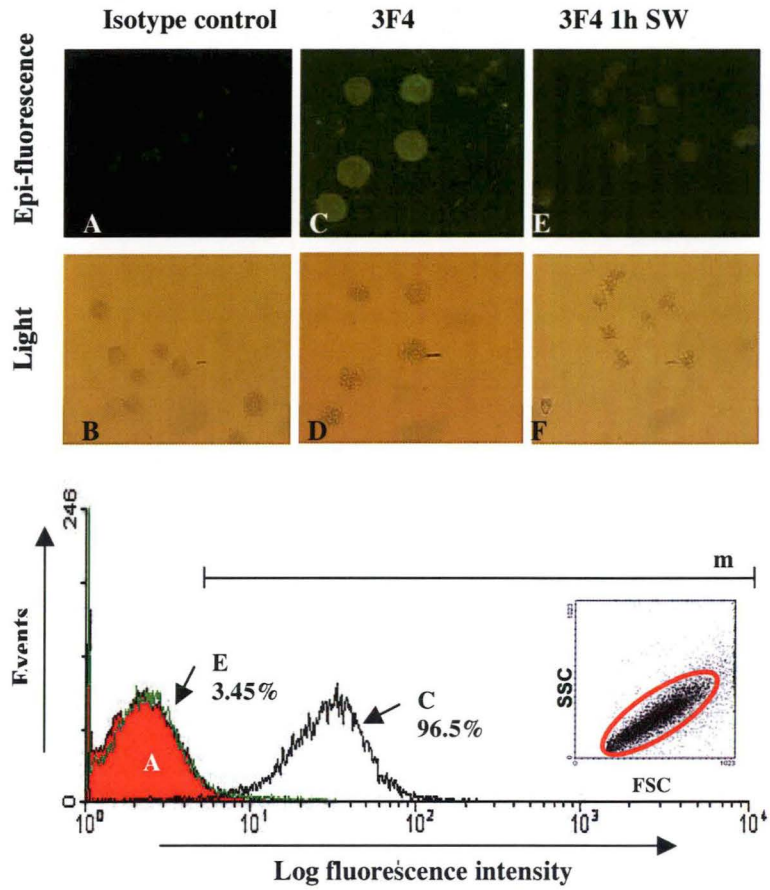


**Figure A3.1.** The monoclonal anti-Gal/GalNAc inhibitable lectin antibody 3F4 was not detectable on the cell-surface of wild-type *Neoparamoeba* spp. after probed cells were incubated in sea water for 1h. Live wild-type *Neoparamoeba* spp. were probed with murine IgG isotype control (A) or 3F4 (C and E). The light micrographs correspond to the adjacent epi-fluorescent images. A sub-sample from each treatment was photographed before quantitative analysis of the remaining sample by flow cytometry. Therefore the histogram labels correspond to the images above. Live cells were either fixed following incubation with primary antibody (C) or transferred to sea water for 1 h (E) before fixation and detection of bound antibodies. The fluorescence intensity of the murine IgG isotype control is shown in the shaded section (A), the intensity of cells incubated in sea water for 1 h was very similar (E). Binding of 3F4 to live wild-type *Neoparamoeba* spp. produces a fluorescence intensity significantly higher than the isotype control (C). Bound antibodies were detected with FITC-conjugated goat anti-mouse IgG. Data are representative of cells within the gated region shown in the dot plot. The proportion of cells producing a fluorescence intensity significantly higher than the isotype control are presented on the histogram and represent data assessed within the marked region (m). Flow cytometric data were analysed and presented using WinMDI 2.8 software.

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# Appendix 4

# Manuscripts

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Vincent, B. N., Morrison, R. N. and Nowak, B. F., (2006) Amoebic gill disease (AGD)affected Atlantic salmon, Salmo salar L., are resistant to subsequent AGD challenge. Journal of Fish Diseases 29, 549-559

Vincent, B. N., Adams, M. B., Crosbie, P. B. B., Nowak, B. F. and Morrison, R. N., (2007) Atlantic salmon (Salmo salar L.) exposed to cultured gill-derived Neoparamoeba branchiphila fail to develop amoebic gill disease (AGD). Bulletin of the European Association of Fish Pathologists 21, 163-166

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# Detection of serum anti-*Neoparamoeba* spp. antibodies in amoebic gill disease-affected Atlantic salmon

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Amoebic gill disease (AGD)-affected Atlantic salmon sometimes developed a serum antibody response to wild-type *Neoparamoeba* spp. Five of 103 AGD-affected Atlantic salmon sampled possessed detectable antibodies that bound wild-type *Neoparamoeba* spp.. Western blotting revealed two distinctly different binding profiles.

Key words: Western blot, antibody response, teleost, ectoparasite

In Tasmania, amoebic gill disease (AGD) is a serious health problem as recurrent epizootics occur (Munday et al., 1990). At present, freshwater bathing is the only commercially viable treatment for AGD and contributes 10-20% to production costs (Munday et al., 2001). Therefore the development of an alternative to freshwater bathing, such as an AGD vaccine, is a priority for many salmon growers.

There is evidence for the development of a serum antibody response in AGD-affected Atlantic salmon (Vincent et al., 2006; Vincent et al., 2009). Fish immune sera have been used to identify protective antigens of the protozoan fish parasite *Ichthyophthirius multifiis* (Ich) (Hines and Spira, 1974; Clark et al., 1988; Clark and Dickerson, 1997; Wang and Dickerson, 2002). Therefore serum antibodies may be exploited to identify wild-type *Neoparamoeba* spp. antigens expressed *in vivo*. In the current study, sera were screened opportunistically from AGD-affected Atlantic salmon with the aim of identifying antigen(s) specific to

wild-type *Neoparamoeba* spp.. Fish were exposed to wild-type *Neoparamoeba* spp., by co-habitation or by inoculation of the fish holding systems with wild-type *Neoparamoeba* spp., the number of fish assessed and tank conditions are listed in Table 1

Amoebic gill disease (AGD) of Atlantic salmon is predominantly associated with the amphizoic marine amoebae, *Neoparamoeba pemaquidensis* (Young *et al*., 2007a, Young *et al*., 2007b). However, *N. pemaquidensis* and *N. branchiphila* have also been isolated from AGD-affected gill tissues of Atlantic salmon by culture (Dyková *et al*., 2005), therefore gill-derived amoebae are described as wild-type *Neoparamoeba* spp.. Wild-type *Neoparamoeba* spp. were isolated as described by Morrison *et al*., (2004). Clonal strains of cultured *N. pemaquidensis* and *N. branchiphila* tested to date are avirulent (Kent *et al*., 1988; Howard *et al*., 1993; Findlay, 2001; Morrison *et al*., 2005; Vincent *et al*., 2007). Therefore, to discriminate between reactive epitope(s) of cultured and wild-type *Neoparamoeba* spp., two previously characterised clonal strains (Dyková *et al*., 2005) were used as controls for Western blotting. These were *N. pemaquidensis* (NP251002) (Morrison *et al*., 2005) isolated from AGD-affected Atlantic salmon, and *N. branchiphila* (SEDMH1) isolated from the sediment of Macquarie Harbour, Tasmania. Amoebae were maintained on seawater malt yeast agar as described by Crosbie *et al*., (2005).



The binding of Atlantic salmon serum antibodies to cultured or wild-type *Neoparamoeba* spp. antigens was assessed by Western blotting and ELISA following the method described by Vincent *et al*. (2006) with minor modification. $6 \times 10^4$ cell equivalents of wild-type or cultured *Neoparamoeba* spp. antigens were loaded per lane. Atlantic salmon test serum, serum previously identified to contain anti-*Neoparamoeba* spp. antibodies (Vincent *et al*., 2006), or normal Atlantic salmon serum was applied at 1:500 in 1% casein solution (Vector Laboratories, Burlingame, CA., USA) and bound antibodies were detected with rabbit anti-salmon IgM at 1:500 followed by alkaline phosphatase (AP)-conjugated sheep anti-rabbit IgG (Chemicon, Boronia, Australia) at 1:2000. Following transfer, bound antigens on adjacent strips were either oxidised with 20 mM sodium periodate (Merck Pty Ltd., Victoria) and 50 mM sodium borohydride (Sigma-Aldrich) or incubated in 50 mM sodium acetate (pH 4.5, Sigma) following the method outlined by Woodward *et al*., (1985).

Antibodies that bound wild-type *Neoparamoeba* spp. antigens were detected by Western blotting in the sera of 6 of 103 fish and in just 2 of these sera (fish 1 and fish 2) antibodies were measurable in an ELISA. Sero-positive samples included two that were obtained from fish from the UTAS co-habitation tank (fish one and fish two) and three fish from the experiment described by Bridle *et al*., (2005). Serum antibodies of fish one and fish two were specific to wild-type *Neoparamoeba* spp. yet distinctively different binding profiles were

produced by Western blotting. Binding of serum antibodies of fish one produced two bands Mr >200 kDa and serum antibodies of fish two produced a smear across a broad molecular weight range (Fig 1). Sodium periodate oxidation of wild-type antigens was performed to assess the binding of anti-*Neoparamoeba* spp. antibodies to peptide or carbohydrate epitope(s). Antibodies present in the serum of fish one were directed towards epitope(s) that were not sensitive to periodate oxidation while antibodies in the serum of fish two failed to bind periodate-treated wild-type *Neoparamoeba* spp. antigens (Fig. 1). Similarly, antibodies present in the three other sero-positive fish from the experiment described by Bridle *et al.*, (2005) were specific to wild-type *Neoparamoeba* spp. and produced a smear across a broad molecular weight range. These antibodies also failed to bind wild-type *Neoparamoeba* spp. antigens after periodate oxidation (data not shown). Similarly, anti-*Neoparamoeba* spp. antibodies detected in the sera of AGD-affected Atlantic salmon in the study described by Vincent *et al.*, (2006) produced a smear in Western blot. The loss of antibody binding following periodate oxidation, as seen here, is indicative of antibody binding to carbohydrate residues (Woodward *et al.*, 1985). Presentation of a smear by Western blotting is characteristic of antibody binding to carbohydrate residues. For example, antibody binding to the proteoglycan agrin (Groffen *et al.*, 1998), carbohydrate antigens of the mould, *Aspergillus versicolor* (Rylpord *et al.*, 2005) and mucin-like glycoproteins (Hong *et al.*, 2001) produced a smear by Western blotting. Monoclonal antibodies produced against wild-type *Neoparamoeba* sp. are predominately directed towards cell-surface carbohydrate



epitopes (Villavedra *et al.*, 2007). Similarly, results presented here and in the study by Vincent *et al.* (2006) suggest that cell-surface carbohydrate epitope(s) of wild-type *Neoparamoeba* spp. may be immunodominant in Atlantic salmon.

The serum antibodies of fish one bound two high molecular weight epitope(s) of wild-type *Neoparamoeba* spp. These high molecular weight epitope(s) are likely to be peptide(s) as antibody binding was observed after sodium periodate oxidation. In addition to the normal serum controls assessed in parallel with the test sera, sera from a further 44 AGD-naive Atlantic salmon was assessed for presence of natural antibodies that may bind *Neoparamoeba* spp. antigens. Antibodies present in the sera of 44 AGD-naive Atlantic salmon did not bind wild-type or cultured *Neoparamoeba* spp. antigens (data not shown). In some cases, natural antibodies may bind the antigens of pathogens. For example, natural antibodies present in serum of goldfish (*Carassius auratus* L.) bind the A-layer proteins of *Aeromonas salmonicida* (Shnyukov *et al.*, 2002) and natural antibodies in the serum of rainbow trout bind the monogenean *Discocotyle sagittata* (Rubio-Godoy *et al.*, 2003). Serum antibodies from 44 AGD-naive Atlantic salmon did not bind wild-type or cultured *Neoparamoeba* spp. antigens suggesting that natural antibodies do not bind *Neoparamoeba* spp. antigen(s). However, AGD-affected fish assessed in the current study were not sampled prior to exposure to *Neoparamoeba* spp. Therefore it cannot be discounted that the serum antibodies described here may be natural antibodies.

The only samples containing anti-*Neoparamoeba* spp. antibodies with measurable activity according to the method outlined by Ardoosh & Kaattari (1990) were those of fish one and fish two. Due to the larger volume of sera attained from fish two, fish two serum was used as the positive control. Binding of anti-*Neoparamoeba* spp. antibodies present in the serum of fish one produced an optical density similar to the positive control sera (Fig. 2). At a serum dilution of 1:100, the mean optical density produced by the AGD-naïve serum was 0.19±0.00, at the same serum dilution, antibodies present in the serum of fish one and fish two (the positive control sera) produced optical densities of 0.79±0.03, 0.63±0.02 respectively. Whilst the optical density produced at the serum dilution of 1:100 was higher for fish one, anti-*Neoparamoeba* spp., the antibody activity of both fish one and fish two serum was equal at 7.7 units $\mu l^{-1}$ of serum. It is interesting that antibodies present in the sera of five fish were detectable by Western blotting yet in an ELISA, antibody activity was only measurable in the sera of fish one and fish two. The ELISA conditions applied here and by Vincent *et al.* (2006) were the same and in both instances, antibodies were detectable by Western blotting, but not by ELISA. In all cases, negative and positive control sera were included on each plate and titration curves were observed for the positive control sera in each ELISA. Processing of wild-type antigen may damage or eliminate reactive epitope(s). However, each ELISA assay was conducted with an independent aliquot of wild-type *Neoparamoeba* spp. antigen from the same antigen pool. Furthermore, fish two antibodies were measurable by the ELISA and appear to bind the same carbohydrate epitope(s) as the three sera-positive fish from the experiment described by Bridle *et al.* (2005). Given this, the failure to detect antibodies by ELISA suggests that denaturation prior to Western blotting may enhance access to the relevant epitopes or that antibody levels are simply very low.

In summary, 5 of 105 AGD-affected Atlantic salmon developed a serum antibody response towards wild-type *Neoparamoeba* spp. Carbohydrate epitope(s) of wild-type *Neoparamoeba* spp. appear to be immunodominant in Atlantic salmon and the development of anti-peptide antibodies specific to wild-type *Neoparamoeba* spp. is, at this point, an isolated finding. Further characterisation of the high molecular weight peptide epitope(s) is warranted and these epitope(s) are currently under investigation.

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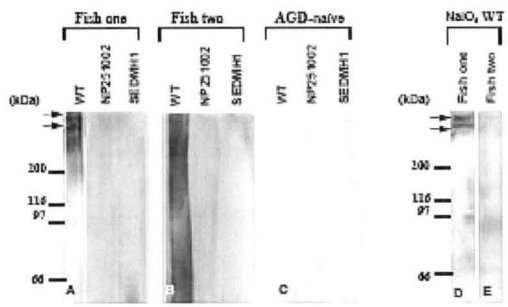


Figure 1. Binding of serum anti-*Neoparamoeba* spp. antibodies from fish one and fish two to wild-type *Neoparamoeba* spp. (WT) produces distinctly different profiles. Anti-*Neoparamoeba* spp. antibodies in fish one serum bind two bands (arrows) >200 kDa (A) of wild-type antigen (WT) and these epitope(s) are not sensitive to periodate oxidation (D). In contrast, binding of anti-*Neoparamoeba* spp. antibodies in fish two serum produce a smear across a broad molecular range (B) and these epitope(s) are sensitive to periodate oxidation (E). Antibodies present in the serum of fish one and fish two do not bind cultured *N. pemaquidensis* (NP251002) or *N. branchiphila* (SEDMH1) and antibodies present in serum from AGD-naive fish do not bind wild-type or cultured *Neoparamoeba* spp. (C).



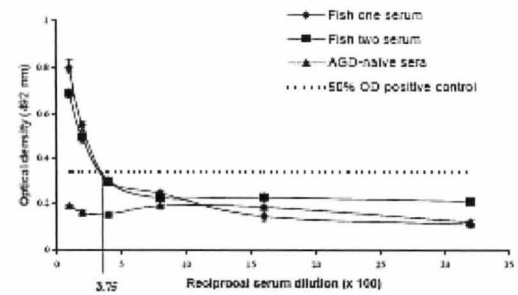


Figure 2. Anti-*Neoparamoeba* spp. antibodies present in sera of fish one and fish two produce a significant optical density in an ELISA. Titration curves represent the mean ± SEM of the optical density at 492 nm. The sera dilution used to calculate the antibody activity of fish one and fish two sera (extrapolated from the optical density (OD) that represented 50% of the OD of the positive sera control) was equal for both fish at 1:375. Antibody activity was calculated by the method described by Arkoosh & Kaattari (1990).

337

338

339 Table 1 Holding conditions of AGD-affected and AGD-naïve Atlantic salmon

340 assessed for the presence of serum anti-*Neoparamoeba* spp. antibodies

341

| Number of fish | Mode of exposure | Days exposed | Salinity | Water Temperature (°C) |
|---|---|---|---|---|
| 17 | Co-habitation | 21-168* | 35 | 16 |
| 23 | inoculation (500 cells $l^{-1}$) | 34 | 35 | 16 |
| 63 | inoculation† (1152 cells $l^{-1}$) | 72 | 35 | 16 |
| 44 | Not exposed | N/A | 0 | 14-16 |

342 †Fish sampled from the experiment described by Bridle *et al.* (2005) *Estimated exposure time.

343

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345

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Manuscript

Cell-surface carbohydrate antigen(s) of wild-type *Neoparamoeba* spp. are immunodominant in sea-cage cultured Atlantic salmon (*Salmo salar* L.) affected by amoebic gill disease (AGD).

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Abstract

A proportion of Atlantic salmon experimentally affected by amoebic gill disease (AGD) develop a serum antibody response to wild-type *Neoparamoeba* spp. These antibodies bind cell-surface epitope(s) and in most cases the epitope(s) are sensitive to sodium periodate oxidation. Here, blood was obtained from triploid and diploid sea-farmed Atlantic salmon after 8, 10 and 13 months of sea-cage culture and assessed for the presence of anti-*Neoparamoeba* spp. (anti-NP) antibodies by Western blot. Generally, an increase in the proportion of fish developing a detectable antibody response to wild-type *Neoparamoeba* spp. was seen over time in culture. The presence of plasma anti-NP antibodies does not appear to be associated with the level of gross gill pathology. Reflected by the number of freshwater bath treatments administered, the triploid and diploid fish may have been affected by AGD multiple times. While the interval between bath treatments increased over time in culture this corresponded to the seasonal reduction in water temperature at the culture site. A further group of putatively AGD-resistant Atlantic salmon broodstock was sampled at 15 months after transfer to sea and anti-NP antibodies were detected in 81% of these samples. The broodstock did not present any gross gill pathology and had not required freshwater bath treatment for over 250 days. Anti-NP antibodies in all sera-positive fish identified here bound cell-surface carbohydrate antigens yet an antibody titre was not detected in any samples by ELISA. Results presented here provide further evidence for the development of an antibody response in AGD-affected Atlantic salmon and that carbohydrate epitopes of wild-type *Neoparamoeba* spp. are immunodominant in Atlantic salmon.

Keywords:
*Neoparamoeba* spp., AGD, carbohydrate antigen, Atlantic salmon

Introduction

Protection against fish parasites has, in some instances, been associated with serum antibodies. Antibody-mediated protection has predominantly been associated with the ectoparasitic haemoflagellates *Cryptobia salmositica* (Chin and Woo, 2003) and *Trypanosoma carassii* (Lischka, et al., 2000). Resistance to the protozoan parasites *Ichthyophthirius multifiliis* (Ich) and *Cryptocaryon irritans* that cause white spot disease is associated with serum and skin-associated (or mucosal) antibodies (Luo, et al., 2006; Wang and Dickerson, 2002; Xu and Klesius, 2002; Yambot and Song, 2006). Moreover there is evidence to suggest that serum antibodies may be associated with protection against ectoparasites of fish. For example, protection of rainbow trout against the monogenean *Dactylogyrus vastator* (Rubio-Godoy, et al., 2003a; Rubio-Godoy, et al., 2003b) and protection of the tomato clown-fish (*Amphiprion frenatus*) against the ectoparasitic dinoflagellate *Amyloodinium ocellatum* has in some cases been associated with serum antibodies (Cobb, et al., 1998). Cross-protection of goldfish (*Carassius auratus*) immunised with Ich or the non-pathogenic ciliate *Tetrahymena pyriformis* against the ectoparasites *Gyrodactylus gilthdorfi*, *Trichodina* sp., *Ichthyobodo necatrix* and *Chilodonella cyprini* is associated with elevated mucus and plasma antibodies (Ling, et al., 1993). Further, a reduction in metamorphosis of the parasitic larval stage of the freshwater mussel *Lampsilis ovata ovata* is associated with the presence of serum antibodies in largemouth bass, *Micropterus salmoides* (Dodd, et al., 2006).

The obligatory marine amoebae, *Neoparamoeba* spp. cause amoebic gill disease (AGD) of Atlantic salmon (Adams & Nowak, 2004a; Dykova, Nowak, Crosbie, Fiala, Peckova, Adams, Machackova & Dvorakova, 2005). Atlantic salmon appear to develop resistance to AGD in terms of reduced gill pathology (Bridle, et al., 2003; Findlay, et al., 1995; Findlay and Munday, 1998) and increased survival (Vincent, et al., 2006). However, evidence to suggest that a humoral immune response may be protective in Atlantic salmon affected by AGD is lacking. Serum antibodies have been detected in some Atlantic salmon demonstrating resistance to AGD in terms of increased survival and/or low-level gill pathology (Vincent, et al., submitted; Vincent, et al., 2006).

Atlantic salmon cultured in Southern Tasmania, Australia, are affected by AGD and the disease is most prevalent during summer months (Adams and Nowak, 2001; Clark and Nowak, 1999). To alleviate this condition, fish are treated with fresh water and this treatment may be repeated multiple times throughout the grow-out period. Atlantic salmon that are experimentally exposed to *Neoparamoeba* spp. for a prolonged period and/or exposed multiple times develop a serum antibody response (Vincent, et al., submitted; Vincent, et al., 2006) and this suggests that sea-cage cultured Atlantic salmon may develop a serum antibody response to *Neoparamoeba* spp. over the culture period. Antibodies that bind cultured *Neoparamoeba* sp. antigen have been detected in the serum of sea-farmed Atlantic salmon (Gross, et al., 2004) however the reactivity of these antibodies with wild-type antigen was not determined. During the current study, blood from sea-farmed Atlantic salmon was screened for anti-*Neoparamoeba* spp. antibodies to 1) identify if cultured Atlantic salmon developed an antibody response to wild-type *Neoparamoeba* spp. and, if so, 2) to use

99 these sera to identify potential vaccine candidates. Results presented here indicate that
100 cultured Atlantic salmon develop an antibody response to wild-type *Neoparamoeba*
101 spp. and antibodies are directed towards cell-surface carbohydrate epitope(s).
102
103 **Materials and methods**
104 *Fish history and sampling*
105 The gross sign of AGD in Atlantic salmon is raised white patches on gill surfaces.
106 AGD-like lesions assessed by gross observation are in many cases associated with
107 *Neoparamoeba* spp., however assessment may be over-estimated as some AGD-like
108 lesions are not associated with *Neoparamoeba* spp. (Adams, et al., 2004). Based on
109 this gross sign, the Huon Aquaculture Company Pty. Ltd. (HAC) in Dover, Tasmania,
110 applies a scoring system as a presumptive diagnosis of the level of AGD. Assessment
111 of AGD-like lesions by gross observation is described as clear, with a gill score of 0,
112 to heavy, that is assigned a gill score of 3 (Adams and Nowak, 2003). On-farm
113 monitoring of AGD is regularly performed by assessing the gross gill pathology of a
114 sub-population of fish from each pen. Freshwater bath treatments are administered
115 when the average gill score for the pen approaches a level of light-medium with a
116 gross gill score above 1.5. Freshwater bathing involves the transfer of fish to an
117 adjacent pen containing a layer of fresh water for 3-4 hours. A total of 375 fish were
118 sampled from HAC. These fish included sea-cage cultured triploid and diploid
119 Atlantic salmon and a group of broodstock that demonstrated resistance to AGD in
120 terms of low, or no, gross gill pathology. The broodstock were the first progeny of a
121 group of Atlantic salmon that were previously selected by HAC as being putatively
122 resistant to AGD in terms of gross gill pathology. Fish were further selected from this
123 population on the basis of gross gill pathology on 2 occasions and fish showing the
124 gross sign of AGD were removed from the population. The brood stock was
125 maintained at the same farm site as commercial culture pens that required freshwater
126 bathing for AGD multiple times (J. Wells, HAC, pers. com.). Induction of triploidy
127 does not guarantee 100% success (Galbreath, et al., 2006) and while this group of fish
128 is termed triploid, testing to confirm this was not carried out and a mixed ploidy
129 population may exist. Table 1 summarises the number of months of sea-cage culture
130 at the time of sampling, sampling month, pen allocation, number of freshwater baths
131 administered, days elapsed post-last bath, average water temperature for the month of
132 sampling, number of fish that were sea-pathogen and the total number of fish sampled
133
134 Blood was taken from triploid and diploid Atlantic salmon on 3 occasions, after 8, 10
135 and 13 months of sea-cage culture. The triploid fish were maintained in two
136 independent pens (pens 1 and 2) and 10 fish from each pen was sampled on each
137 occasion. The diploid fish were initially contained in a single pen (pen 3) and 20 fish
138 from this pen were sampled after 8 and 10 months of sea-cage culture. Following the
139 second sampling at 10 months, the diploid fish were split across two pens (pens 3a
140 and 3b) and for the final sampling at 13 months, 10 fish from each pen were sampled.
141 Blood was taken from 55 of the 75 AGD-resistant fish on one occasion after they had
142 been in sea-cage culture for 15 months. Triploid and diploid Atlantic salmon were
143 terminally anaesthetised while the AGD-resistant fish were anaesthetised for blood
144 sampling only and in all cases fish were anaesthetised using clove oil (0.02% w/v).
145 Blood was taken from the caudal vein and stored in heparinised (triploid and diploid)
146 and non-heparinised tubes (AGD-resistant) on ice overnight. The following day,
147 blood was centrifuged at 1000×*g* for 10 min and the plasma (triploid and diploid) and
148 serum (AGD-resistant) was stored at -20°C. The variation in blood collection methods

described here was due to the samples from the triploid and diploid fish originating from an independent study where plasma had been collected.

*Detection of anti-Neoparamoeba spp. antibodies*

*Cultured and wild type amoebae*

Wild-type amoebae were isolated as described by Morrison et al., (2004) from AGD-affected Atlantic salmon housed at the University of Tasmania aquaculture centre. As all cultured *Neoparamoeba* spp. tested to date are axenic (Kent, et al., 1988; Morrison, et al., 2005) two previously characterised clonal strains (Dykova et al., 2003) of *Neoparamoeba* spp. were used to discriminate between reactive epitope(s) of wild-type and cultured *Neoparamoeba* spp.. These were *Neoparamoeba pemaquidensis* (NP251002) (Morrison, et al., 2005) isolated from AGD-affected Atlantic salmon, and *Neoparamoeba branchiphila* (SEDVH8) isolated from sediment of Macquarie Harbour, Tasmania. Amoebae were maintained on sea water malt yeast agar; 75% (v/v) coarse-filtered sea water (35‰), 25% (v/v) distilled water, 0.01% (w/v) malt, 0.01% (w/v) yeast (Gistod. Hampshire, England), 2% (w/v) Bacto agar (Bacto, Dickson & Co., Sparks, Maryland, USA). Cells were harvested by washing the agar with sterile sea water using a transfer pipette. Wild-type and cultured cells were concentrated by centrifugation at 500× *g* for 5 min and enumerated by haemocytometer. For Western blot and ELISA assays amoebae were washed twice with phosphate buffered saline (PBS, pH 7.2) and the cell pellet was stored at -80° C.

*SDS-PAGE and Western blot*

Binding of plasma (triploid and diploid) and serum (AGD-resistant) antibodies to cultured and wild-type *Neoparamoeba* spp. was assessed by Western blot. Amoebae antigens were reduced in buffer containing β-mercaptoethanol by boiling for 10 min and separated through 6% polyacrylamide gels with $8 \times 10^4$ cells loaded in each lane (12.4 µg total protein/lane). Antigens were transferred to nitrocellulose membrane (Hybond-C extra, Amersham Biosciences, Little Chalfont, UK) using a semi-dry transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA) and membranes were blocked in casein solution (Vector, Burlingame, CA, USA). Blocking and antibody incubation steps were for 30 min and in between incubation steps, membranes were washed 3× 4 min with tris-buffered saline (TBS, pH 7.2). Following the final antibody incubation, membranes were washed 3× in TBS and then in 0 M tris (pH 9.5) for 5 min. All incubation and wash steps were conducted at room temperature. Initial screening of the 175 samples was conducted using pooled serum and plasma. Pools consisting of serum or plasma from 5 to 7 fish were incubated at 1:100 with membrane strips (single lane) of wild-type *Neoparamoeba* spp. antigen. Bound antibodies were detected with rabbit anti-salmon IgM at 1:500 (kind gift from Dr. D. Zilberg), sheep anti-rabbit alkaline phosphatase (AP) (Chemicon, Boronia, Australia) at 1:1000 and developed with BCIP/NBT (Sigma, St Louis, Missouri, USA) following the manufacturer's instructions. Binding of the polyclonal rabbit anti-salmon IgM to the heavy chain of Atlantic salmon IgM has been previously described (Vincent, et al., 2006). Pooled samples that returned a positive Western blot result were subsequently screened individually as described above. In parallel, normal salmon plasma and serum pooled from 5 fish held in fresh water and therefore AGD-naïve was included as negative controls. Finally, positive samples were pooled (5 per pool) and assessed for antibody binding to cultured amoebae antigens. As the detection limit using chemiluminescence is more sensitive than BCIP/NBT, pooled positive serum and plasma were incubated at 1:500 and bound antibodies were

199 detected with rabbit anti-salmon IgM at 1:100, sheep anti-rabbit AP at 1:2000 and
200 enhanced chemiluminescence (ECL) using DuoLuX (Vector, Burlingame, CA, USA),
201 Kodak BioMax Light Film and Kodak GBX developing and fixing reagents (Sigma,
202 Castle Hill, NSW, Australia) following the manufacturer's instructions. Binding of
203 anti-*Neoparamoeba* spp. antibodies identified here was also assessed against cultured
204 and wild-type antigens that were separated through a 12% polyacrylamide gel to
205 identify binding to antigens of a lower molecular weight.
206
207 *Sodium periodate oxidation of carbohydrate epitope(s) of wild-type Neoparamoeba*
208 *spp.*
209 Wild-type *Neoparamoeba* spp. antigens were transferred to nitrocellulose membrane
210 as outlined above and from the same membrane, bound antigens on adjacent strips
211 were either oxidised with 20 mM sodium periodate (Merck Pty Ltd., Victoria) and 50
212 mM sodium borohydride (Sigma-Aldrich, St Louis, Missouri, USA) or incubated in
213 50 mM sodium acetate (Sigma-Aldrich) pH 4.5, following the method outlined by
214 Woodward et al., (1985). Membranes were then washed 3× with PBS, blocked,
215 probed and developed by ECL as outlined above.
216
217 *Enzyme-linked immunosorbent assay (ELISA), immunocytochemistry and flow*
218 *cytometry.*
219 Binding of anti-*Neoparamoeba* spp. antibodies found in serum or plasma by Western
220 blot was assessed individually by ELISA. Optimal conditions for ELISA were
221 determined empirically and have been described previously (Vincent et al., 2006). For
222 immunocytochemistry and flow cytometry, wild-type amoebae were isolated from
223 AGD-affected Atlantic salmon as outlined above. Cells were fixed in seawater
224 Davidson's for 1 h at room temperature (RT) and washed 4× with PBS. Amoebae
225 were placed in the wells ($5 \times 10^5$ cells/well) of U-bottomed 96-well microplates
226 (Sarstedt, Inglis Farm, South Australia) and blocked in 0.1% BSA-PBS for 30 min at
227 4°C. Cells were probed with normal Atlantic salmon serum (pooled from 5 fish held
228 in fresh water and therefore AGD-naïve) and a representative pool of salmon anti-
229 *Neoparamoeba* spp. serum (5 fish) that tested positive for anti-*Neoparamoeba* spp.
230 antibodies by Western blot. Cells were incubated with salmon serum at 1:10 (BSA-
231 PBS) and bound antibodies were detected with rabbit anti-salmon IgM at 1:100 and
232 sheep anti-rabbit FITC (Chemicon, Melbourne, Australia) at 1:50. Cells were washed
233 3× with PBS following each antibody incubation and photographed (Leica DC300F,
234 Leica Microsystems, Wetzlar, Germany) using light and fluorescent microscopy. The
235 proportion of wild-type *Neoparamoeba* spp. expressing epitope(s) to which the serum
236 anti-*Neoparamoeba* spp. antibodies bound was quantified by flow cytometry (Coulter
237 Epics, Beckman Coulter, USA). A minimum of $10^4$ events were assessed per
238 treatment and data were analysed using WinMDI 2.8 software (Joseph Trotter,
239 Scripps Research Institute, La Jolla, CA, USA).
240
241 Results
242 Atlantic salmon assessed in the current study for anti-*Neoparamoeba* spp. (anti-NP)
243 antibodies were first sampled when they had been in sea-cage culture for 8 months.
244 From the 20 triploid and 20 diploid fish sampled at this time, anti-NP antibodies were
245 detected by Western blot in 5 samples taken from the diploid fish (Fig. 1). After 10
246 months in sea-cage culture, anti-NP antibodies were detected in samples taken from 8
247 of the 20 triploid fish (3 from pen 1 and 5 from pen 2). An increase in the proportion
248 of diploid fish that developed a detectable antibody response to wild-type

249 *Neoparamoeba* spp. was seen with 17 of the 20 diploid fish sampled testing positive
250 for anti-NP antibodies. A further increase in the proportion of triploid Atlantic salmon
251 with detectable anti-NP antibodies was observed after 13 months of sea-cage culture
252 and 14 (5 from pen 1 and 9 from pen 2) of the 20 triploid fish sampled had developed
253 an antibody response to wild-type *Neoparamoeba* spp.. In contrast, after 13 months in
254 culture fewer positive samples from the diploid fish were identified with 9 samples (4
255 from pen 3a and 5 from pen 3b) testing positive for anti-NP antibodies by Western
256 blot. The putatively resistant Atlantic salmon broodstock had been maintained at sea
257 for 15 months at the time of sampling. Fifty-five of the 75 fish in the broodstock
258 population were sampled and Western blot analysis identified 45 of these fish had
259 developed an antibody response to wild-type *Neoparamoeba* spp..
260
261 After 8 months in sea-cage culture, the gross gill assessment of the triploid and
262 diploid fish sampled was predominantly 0 (clear-very light). The gross pathological
263 sign of AGD was more pronounced in some fish that were sampled after 10 months in
264 sea-cage culture. The gross gill assessment of sero-positive and sero-negative fish
265 ranged from 0 to 3 (heavy) and the majority of sero-positive diploid fish displayed a
266 light level of AGD with a gross gill score of 1 (Fig. 2a). Gross gill scores of sero-
267 positive triploid fish from pen 1 ranged between 0 and 2 while in pen 2 scores ranged
268 from 0 to 3. After 13 months of sea-cage culture, the majority of triploid and diploid
269 fish assessed as having a clear to very light AGD and were assigned a gross gill score
270 of 0 to 1. No diploid fish displayed moderate-heavy AGD as the highest gill score
271 assigned was 1. While there was a higher proportion of sero-positive triploid fish with
272 a gross gill score of 0, the proportion of sero-positive and sero-negative diploid fish
273 with a gross gill score of 0 and 1 was similar. No triploid or diploid fish sampled after
274 13 months in sea-cage culture displayed heavy AGD infection as the highest gill score
275 assigned was 2. (Fig 2b). Gross gill scores of sero-positive triploid fish from pen 1
276 ranged between 0 and 1 while in pen 2 scores ranges from 0 to 2. Tentatively
277 AGD-resistant broodstock had not been reared with fresh water for AGD for over 250
278 days and the gross gill assessment prior to sampling was recorded as clear for all fish.
279
280 The Western blot profile produced by binding of plasma anti-NP antibodies present in
281 samples from the triploid and diploid Atlantic salmon was a smear. Similarly, binding
282 of antibodies present in serum taken from the broodstock produced a smear from
283 around 45 kDa to > 200 kDa. Further assessment of antibody binding after antigens
284 were separated through a 12% gel showed that below 45 kDa less anti-NP bound to
285 the amoebae antigens and no binding was seen below 31 kDa (data not shown). As the
286 Western blot binding profile produced by anti-NP antibodies detected in the
287 above mentioned samples was consistent, the remaining analysis was performed using
288 a pooled sub-group (n=5) of serum taken from the broodstock and will be from here
289 on referred to as salmon anti-NP serum.
290
291 Salmon anti-NP antibodies did not bind cultured *Neoparamoeba* sp. antigen and no
292 binding occurred following periodate oxidation of wild-type antigen, suggesting that
293 these antibodies are directed towards carbohydrate epitope(s) of wild-type
294 *Neoparamoeba* spp. (Fig. 3). Binding of salmon anti-NP antibodies to wild-type
295 *Neoparamoeba* spp. was quantified by flow cytometry, producing a fluorescence
296 intensity significantly higher than the normal serum control (Fig. 4). While binding
297 was detected by Western blot and flow cytometry, salmon anti-NP serum failed to

298 produce an optical density in excess of the normal serum control in an ELISA (data
299 not shown).
300
301 **Discussion**
302 In some cases, sea-farmed Atlantic salmon develop an antibody response to wild-type
303 *Neoparamoeba* spp. and anti-NP antibodies detected in the current study are directed
304 towards cell-surface carbohydrate epitope(s). Antibodies that bind cell-surface
305 carbohydrate epitope(s) of wild-type *Neoparamoeba* spp. have also been detected in
306 some Atlantic salmon experimentally inoculated with wild-type *Neoparamoeba* spp.
307 (Vincent, et al., submitted; Vincent, et al., 2006). In addition, monoclonal antibodies
308 produced against cell-surface antigens of wild-type *Neoparamoeba* spp. are
309 predominantly directed towards carbohydrate epitope(s) (Villavedra, et al., In press).
310 Together, these results suggest that cell-surface carbohydrate epitopes of wild-type
311 *Neoparamoeba* spp. are immunodominant. Carbohydrate antigens are abundant on the
312 cell-surface of many protozoan parasites and some of these structures are important
313 for attachment to the host (Mendonça-Previato, et al., 2005). While the mechanism(s)
314 that mediate attachment of *Neoparamoeba* spp. to Atlantic salmon gill tissue are
315 unknown, the dominance of cell-surface carbohydrate epitope(s) suggests that
316 attachment may be mediated by cell-surface glycoconjugates.
317
318 While anti-NP antibodies have been detected in many fish, both in the current and
319 previous studies (Vincent, et al., submitted; Vincent, et al., 2006), antibody titre in the
320 majority of cases, was not detectable in an ELISA. The same Western blot binding
321 profile (smear) as reported in the current study was produced by binding of anti-NP
322 antibodies present in serum of some Atlantic salmon that were exposed to wild-type
323 *Neoparamoeba* spp. naïve (Vincent, et al., 2006) and anti-NP antibodies present in the
324 serum of 4 fish that were exposed to wild-type *Neoparamoeba* spp. for a prolonged
325 period (Vincent, et al., submitted). Despite producing similar Western blot binding
326 profiles, a significant serum anti-NP antibody titre was only measured by an ELISA in
327 one of these samples (Vincent, et al., submitted). This suggests that failure to measure
328 antibody titre by an ELISA in the current study or the study by Vincent et al., (2006)
329 is not associated with the ELISA conditions but rather with very low antibody levels.
330 Wild-type *Neoparamoeba* spp. are occasionally entrapped in intracellular vesicles
331 containing lysosome-like cells (Adams and Nowak, 2001) and antigen processing may
332 be facilitated by MHC II$^+$ cells present in AGD gill lesions (Morrison, et al., 2006).
333 The level of antigen processing may therefore be restricted by the number of
334 *Neoparamoeba* spp. that become entrapped, perhaps influencing serum anti-NP
335 antibody titre. Results presented here suggest that the presence of low-level plasma
336 anti-NP antibodies does not appear to be related to the level of gross gill pathology.
337 After 10 months of sea-cage culture there was a substantially higher proportion of
338 sero-positive diploid fish with a gross gill score of 1, however sero-positive and sero-
339 negative fish from both triploid and diploid populations were represented across the
340 range of gross gill pathology with scores from 0 to 3. An overall reduction in gill
341 pathology was seen after 13 months with no fish displaying heavy AGD and although
342 no sero-positive diploid fish were assessed higher than a gill score of 1, the proportion
343 of sero-negative and sero-positive diploid fish with gill scores of 0 and 1 were similar.
344 There was a slight variation in the level of gross pathology of the sero-positive fish
345 between triploid pens 1 and 2 and the diploid pens 3a and 3b however little can be
346 concluded from this due to the low sample size. As low parasite numbers are
347 associated with high-titre specific antibody in cultured rainbow and brown trout

348 affected by the monogenean *Dactylogyrus sagittatus* (Rubio-Godoy, et al., 2003a), the
349 presence of high-titre anti-NP antibodies may influence the development of AGD-like
350 gill pathology.
351
352 The number of fish that developed an anti-NP response increased over time with the
353 exception of the diploid fish at the final sampling after 13 months in culture. As only
354 10 fish were sampled from each cage, the decline in the number of sero-positive
355 diploid fish identified at the final sampling may have been influenced by the low
356 numbers of fish sampled. It has been hypothesised that the duration of, or multiple
357 exposure to, wild-type *Neoparamoeba* spp. may influence the development of an
358 antibody response in Atlantic salmon (Vincent, et al., submitted; Vincent, et al., 2005)
359 and data presented here provide further support for this. Similarly, the development of
360 a serum antibody response to the ectoparasitic copepod, *Lepeophtheirus salmonis*
361 appears to be influenced by the duration of exposure and/or parasite abundance.
362 Rainbow trout naturally exposed to a low-level *L. salmonis* infection for 8 weeks
363 (Grayson, et al., 1995) and Atlantic salmon exposed to a moderate *L. salmonis*
364 infection for 12 months do not develop a serum antibody response (MacKinnon,
365 1993). However, Atlantic salmon exposed to a high-level *L. salmonis* infection for up
366 to 2 years develop a serum antibody response (Grayson, et al., 1991). Similarly for
367 AGD-affected Atlantic salmon, the level of infection and potentially, the subsequent
368 increase in antigen processing events may influence the development of a serum
369 antibody response in AGD-affected Atlantic salmon.
370
371 The time elapsed between freshwater bath treatments increased over time in culture
372 with the exception of one pen of diploid fish. An increase in the period between
373 freshwater bathing for AGD may be interpreted as an indication of resistance.
374 However, environmental conditions including increased salinity and temperature are
375 key factors that influence AGD (Adams and Nowak, 2003; Adams and Nowak, 2004;
376 Clark and Nowak, 1999). The greatest period between freshwater bath treatments
377 occurred between the second and third sampling that coincided with the period
378 between late summer and late autumn where a seasonal reduction in seawater
379 temperature occurred. Freshwater bathing is conducted on the basis of gross gill
380 pathology and gill score data collected after 13 months in sea-cage culture suggests
381 that at this time fish were experiencing a lower level of AGD. The putatively AGD-
382 resistant Atlantic salmon broodstock did not require a freshwater bath for over 8
383 months yet the development of a serum antibody response in a large proportion of
384 these fish indicates that they were exposed to *Neoparamoeba* spp.. These fish were
385 housed in a single sea-cage at a very low stocking density and this may have
386 contributed to the low-level of infection. Alternatively, as these Atlantic salmon were
387 the first progeny of broodstock that were previously selected by HAC on the basis of
388 gross gill pathology, resistance to AGD in terms of gross gill pathology may be
389 inherent.
390
391 There are many challenges associated with the development of anti-parasite vaccines
392 and this is reflected by the few anti-parasite vaccines commercially available.
393 Vaccines for the cattle tick, *Boophilus microplus*, were introduced in 1994 and are
394 currently the only commercially available ectoparasite vaccines (Nuttall, et al., 2006).
395 Identification of protective peptide antigens is important for the development of an
396 economically viable AGD vaccine. To date, recovery of salmon anti-NP antibodies to
397 putative peptide epitope(s) remains an isolated case (Vincent, et al., submitted) and

carbohydrate epitope(s) are predominantly recognised by Atlantic salmon serum anti-NP antibodies. In addition, monoclonal antibodies produced against desialoganglioside wild-type cell-surface antigens react with very few cell-surface peptide epitope(s) that are unique to wild-type *Neoparamoeba* spp (Villavedra, et al., In press). Together, this suggests that the identification of peptide candidate vaccine antigens by screening serum against wild-type *Neoparamoeba* spp is unlikely. Mucosal antibodies have been associated with resistance of fish against protozoan parasites such as Ich (Wang and Dickerson, 2002; Xu and Klesius, 2002) and *Cryptocaryon irritans* (Luo, et al., 2006; Yambot and Song, 2006). While anti-NP antibodies were not detected in cutaneous mucus of putatively AGD-resistant Atlantic salmon (Vincent, et al., 2006), resistance of Atlantic salmon to *Neoparamoeba* spp. may be associated with a more localised response. The potential that a more localised antibody response, in the gill mucus or epithelium, may play a role in resistance of Atlantic salmon to AGD warrants further investigation.

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Table(s)

Table 1. Sea-cage cultured Atlantic salmon assessed for anti-*Neoparamoeba* spp. antibodies in the current study required multiple freshwater baths for AGD over the grow-out period. Putatively AGD-resistant broodstock were not treated for AGD for over 210 days. Triploid fish were maintained in two independent pens throughout the sampling period while the diploid fish were initially held in a single pen and were split across two cages after 10 months in sea-cage culture. These data summarise the number of months of sea-cage culture at the time of sampling, sampling month and average sea water temperature, pen allocation, number of freshwater baths administered, days elapsed post-last bath, number of sero-positive fish and the total number of fish sampled.

| Group | Months in sea-cage culture | Sampling month and average water temperature* (°C ± SE) | Pen allocation | Total Freshwater baths | Days post-last bath | Number Sero-positive/total fish sampled |
|---|---|---|---|---|---|---|
| AGD-affected Triploid | 8 | December 15.5 (0.2) | 1 | 4 | 23 | 0/10 |
| | | | 2 | 4 | 16 | 0/10 |
| | 10 | February 17.1 (0.2) | 1 | 4 | 77 | 3/10 |
| | | | 2 | 4 | 66 | 5/10 |
| | 13 | May 12.6 (0.1) | 1 | 5 | 104 | 5/10 |
| | | | 2 | 5 | 101 | 9/10 |
| AGD-affected Diploid | 8 | December 15.5 (0.2) | 3 | 4 | 10 | 5/20 |
| | 10 | February 17.1 (0.2) | 3 | 4 | 60 | 17/20 |
| | 13 | May 12.6 (0.1) | 3a | 5 | 95 | 4/10 |
| | | | 3b | 6 | 15 | 5/10 |
| Putative AGD-resistant broodstock | 15 | March 15.4 (0.1) | NA | NA | >230 | 45/51 |

* Average seawater temperature for the month of sampling taken at a depth of 1m.

Figure(s)

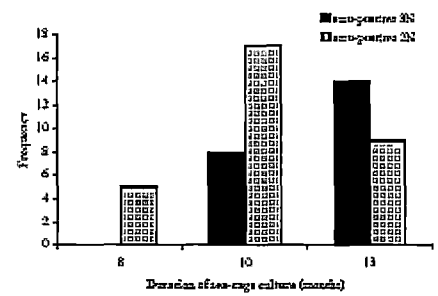


Figure 1. Antibodies that bind wild-type *Neoparamoeba* spp. were detected by Western blot in the plasma of sea-cage cultured triploid and diploid Atlantic salmon. The proportion of triploid and diploid Atlantic salmon that developed an anti-*Neoparamoeba* spp (anti-NP) antibody response increased after 10 months in sea-cage culture. A further increase in the proportion of triploid fish with detectable anti-NP antibodies was seen after 13 months in sea-cage culture. In contrast, a decline in the proportion of diploid fish with detectable anti-NP antibodies was seen after 13 months. The frequency of sero-positive diploid (2N) and triploid fish (3N) are presented. Samples from 20 diploid and 20 triploid fish were assessed by Western blot at each sampling

Figure(s)

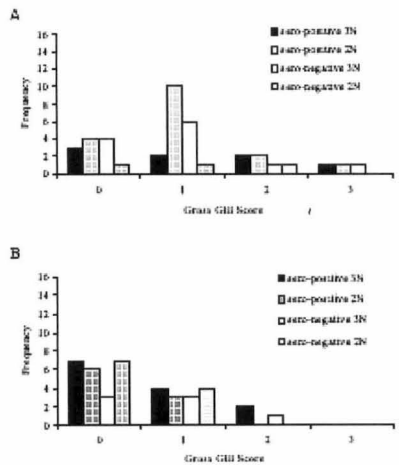


Figure 2. Assessment of AGD-like lesions on gills of Atlantic salmon from triploid and diploid populations ranged from clear to heavy in both sero-positive and sero-negative fish. After 10 months in culture (A), a greater proportion of sero-positive diploid Atlantic salmon presented with light AGD infection by gross observation. An overall higher proportion of fish presenting with clear-very light gross gill pathology was seen after 13 months in culture (B) and no fish sampled at this point were assessed with heavy AGD pathology. The frequency of sero-positive and sero-negative fish assigned the gross gill scores of 0 (clear-very light), 1(light), 2 (moderate) and 3 (heavy) are presented. Samples from 20 diploid and 20 triploid fish were assessed at each of the 3 sampling occasions.

Figure(s)

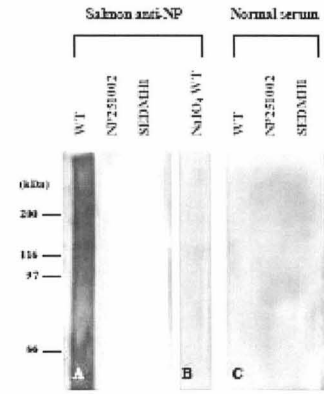


Figure 3. Binding of salmon anti-*Neoparamoeba* spp. (anti-NP) antibodies produces a smear across a broad molecular weight range (*A*) and reactive epitope(s) are sensitive to periodate oxidation (B). Anti-NP antibodies bind wild-type *Neoparamoeba* spp. (WT) and do not bind cultured *Neoparamoeba pemaquidensis* (NP251002) and *Neoparamoeba branchiphila* (SEDMH1) (*A*) and antibodies present in normal Atlantic salmon serum from AGD-naive fish do not bind wild-type or cultured *Neoparamoeba* spp. (C). Antigens were reduced in sample buffer containing β-mercaptoethanol, separated through a 6% polyacrylamide gel and each lane was loaded with $8 \times 10^4$ cells (12.4 µg total protein). Wild-type antigens were transferred to nitrocellulose and treated with sodium periodate ($NaIO_4$,WT) (B). Membranes were probed with pooled (5 fish) salmon anti-NP (*A*) and normal Atlantic salmon serum (C). Bound antibodies were detected with rabbit anti-salmon IgM, sheep anti-rabbit AP and chemiluminescence. Normal serum was taken from Atlantic salmon held in fresh water and the fish were therefore AGD-naive.

Figure(c)

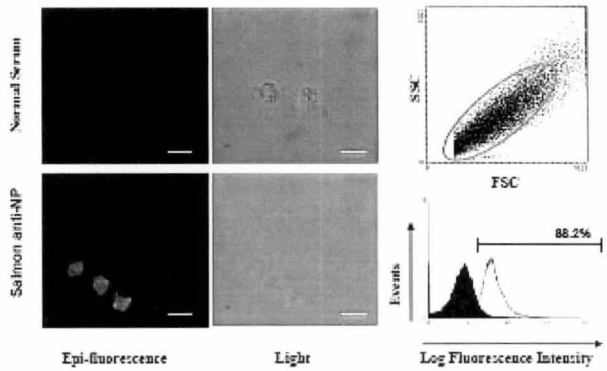


**Figure 4.** *Anti-Neoparamoeba* spp. (anti-NP) antibodies bind cell-surface epitope(s) of wild-type *Neoparamoeba* spp. producing a fluorescence intensity significantly higher than the normal serum control. Wild-type *Neoparamoeba* spp. were fixed and probed with normal salmon serum (Normal serum, top panel) or anti-NP serum (Salmon anti-NP, lower panel). Adjacent images are the corresponding light micrographs. Bound antibodies were detected with rabbit anti-salmon IgM and sheep anti-rabbit FITC. Normal serum and salmon anti-NP serum was pooled from 5 fish. Normal serum was taken from Atlantic salmon held in fresh water and these fish were therefore AGD-naïve. Flow cytometric data presented include cells within the gated region shown in the dot-plot (top right) and the shaded area of the histogram represents cells probed with normal salmon serum (lower right). Flow cytometric data were analysed and presented using WinMDI 2.8 software. Scale bars = 50 μm